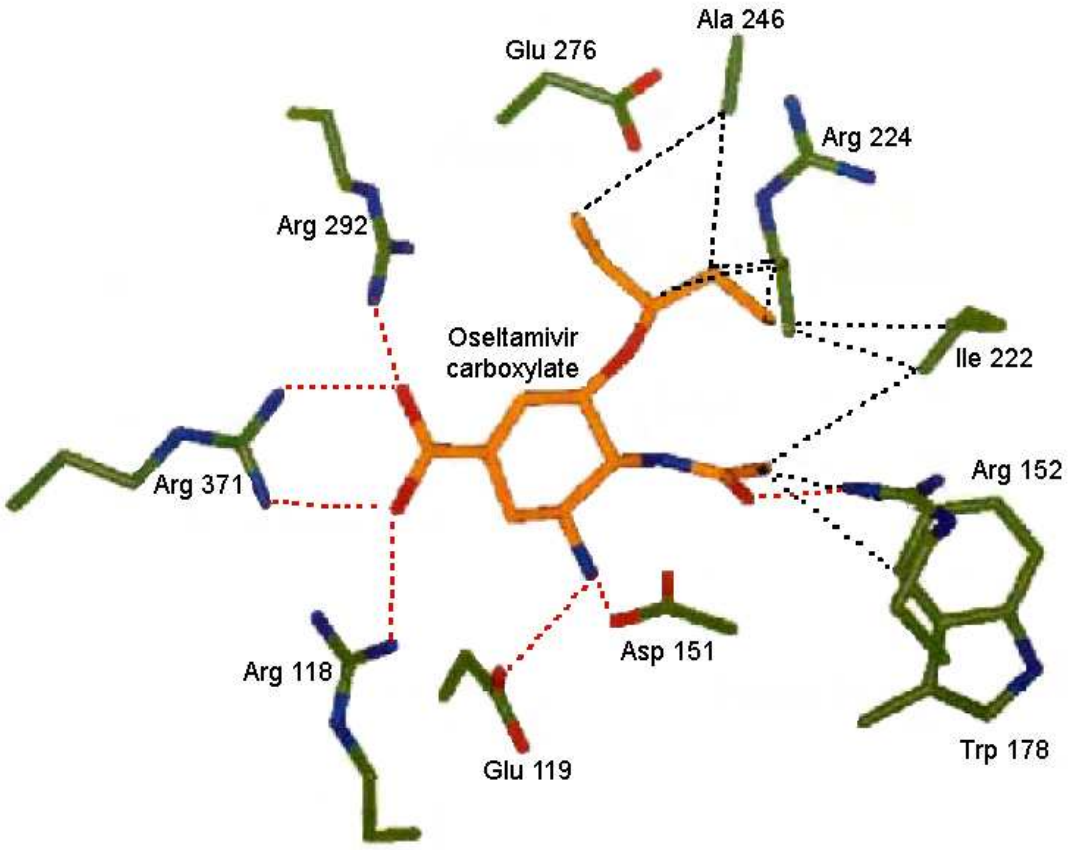
Thesis for the degree of *Candidata Pharmaciae* 2006

OSELTAMIVIR CARBOXYLATE PRODRUGS

Studies around potential coumarin prodrugs of the neuraminidase inhibitor oseltamivir carboxylate



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ABSTRACT

Influenza is an acute respiratory disease causing hundreds of thousands hospitalized each year worldwide. Vaccination plays a major role in the prevention of influenza, but due to constant antigenic drift and the ever-present potential for antigenic shift of the virus and an associated pandemic, antiviral therapy has an important role in the management of influenza.

Oseltamivir (Tamiflu®) is the ethyl ester prodrug of oseltamivir carboxylate, a potent and selective inhibitor of the influenza virus enzyme neuraminidase. Oseltamivir is extensively converted to oseltamivir carboxylate by hepatic esterases and eliminated entirely in the urine. Neuraminidase is a surface glycoprotein of influenza viruses that cleaves terminal sialic acids from carbohydrates. This enzyme is critical for viral release from infected cells, prevents virus aggregation and facilitates viral spread in the respiratory tract.

In this thesis it was synthesized a few novel promoieties from coumarin, which could be used as potential amine prodrugs of oseltamivir carboxylate. Different strategies for these synthesis were studied and attempted. By synthesizing these prodrugs the binding to albumin might increase and a passive targeting effect to inflamed tissue can be obtained. Synthesizing different esters of *cis*-2-hydroxycinnamic acid as pro moiety takes advantages of the fast breakdown from esterases *in vivo* and a spontaneous lactonization which regenerates the coumarin unit. Since coumarin has been studied for many years, the toxicity profile is well known, and it is found be nontoxic. The conversion or activation of a prodrug to the parent drug molecule in the body is a result of enzyme mediated cleavage or pH dependent hydrolysis of the established prodrug linkage. The human body is rich in enzymes that are capable of quickly hydrolyzing these ester bonds, they are ubiquitous distributed and several types are found in the blood, liver, and other organs and tissues. The rate of hydrolytic breakdown of prodrug is also dependent of steric effects/hindrance within the pro moiety and the percentage of protein binding.

The active metabolite oseltamivir carboxylate was hydrolyzed from oseltamivir phosphate under acid condition. Crystals was attempted grown from both oseltamivir phosphate and oseltamivir carboxylate to obtain the x-ray crystal structure. The crystals grown were needle shaped and very small, less then 10 μm in diameter. The needle grains had also connected with each other to form a continuous network. To obtain single-crystal x-ray crystallographic data the crystals had to be three times larger and these crystals could therefore not be used.

ABBREVIATIONS

ACN	Acetonitrile
d	Doublet
dd	Double doublet
dt	Double triplet
DANA	2-deoxy-2,3-dihydro-N-acetylneuraminic acid, Neu5Ac2en
DCM	Dichloromethane
DCC	N,N-dicyclohexylcarbodiimide
DMAP	N,N-dimethylpyridin-4-amine
ε	Molar absorptivity
eq	Molar equvivalents
G1	HPLC method, gradient
GS 4071	Oseltamivir carboxylate
GS 4104	Oseltamivir
HA	Hemagglutinin
HPLC	High Performance Liquid Chromatography
HSA	Human serum albumin
I1	HPLC method, isocratic (ACN:H_2O (1:9))
IFN	Interferon
J	Coupling constant
LAH	Lithium aluminum hydride
m	Multiplet
MeOH	Methanol
MS	Mass spectrometry
NA	Neuraminidase
NAI	Neuraminidase inhibitors
NMR	Nuclear magnetic resonance
OTV	Oseltamivir
OTV-C	Oseltamivir carboxylate

PCC	Pyridinium chlorochromate
s	Singlet
ssRNA	Single-stranded RNA
t	Triplet
TBDMS	*tert*-Butyl-dimethylsilyl
td	Triple doublet
TEA	Triethylamine
THF	Tetrahydrofurane
UV	Ultra violet
δ	Chemical shift (ppm)
WHO	World Health Organization

1 TABLE OF CONTENTS

2 INTRODUCTION

2.1 EPIDEMIOLOGY AND HISTORY OF INFLUENZA

Definition of influenza:
*"Influenza is a highly infectious disease, particularly attacking the respiratory system, transmitted by inhalation of micro droplets and causing periodic epidemics and pandemics [**1**]."*

Influenza is an acute respiratory disease which is caused by the influenza virus and occurs mostly during the winter months due to endemic viral disease. The cold, crowding of people and higher humidity in the winter may favor virus survival outside the host airway. The influenza virus usually enters the body through mucous membranes in the mouth, nose or eyes. Then the virus infects the upper and lower respiratory tract: the nose, sinuses, throat, lungs and middle ear. The typically course of influenza disease is a 1-3 day period of incubation, with the following symptoms which are abrupt onset of fever and chill. Associated symptoms of myalgia, malaise, anorexia, dry cough, headache and sore throat are also usual. When a person with influenza coughs or sneezes, the virus becomes airborne and can be inhaled by anyone nearby. Since the virus is highly contagious and transmitted by aerosol and contagion, it spreads rapidly. Influenza afflicts large parts of the population in annual epidemic outbreaks, which are unpredictable in time and severity.

After the discovery of the influenza virus in 1933, research on this pathogen exploded. This resulted in the development of an influenza vaccine. Vaccines protects against influenza by stimulating an immune response in the recipients. The effect of influenza vaccine depends on match between the antigens contained in the vaccine and the circulating viruses. Due to rapid antigenic drift the vaccine composition has to be updated annually. This ensures that the viral strains in the vaccine are closely matched to circulating strains. In response to recognition of the antigenic variability of influenza, the World Health Organization (WHO) established an influenza surveillance program in 1947. This program operates through a network of national and regional centers to monitor global influenza activity. This monitoring ensures that the composition of the influenza vaccines are annual updated and serves as an early warning of new pandemic strains [**2**].

Although vaccination now has become the primary defense for preventing influenza, there are several reasons for why there still is a need for development of novel, selective antiviral drugs for the treatment of influenza. Antigenic drift in the virus may occur after the vaccine has been manufactured and distributed during any influenza season, the vaccine will be less protective, and outbreaks can occur more easily. Also the production of a vaccine against a new strain of influenza virus require several months of preparation. Therefore it is likely that vaccine would not be available for the first wave spread of influenza virus under a pandemic [**3**]. The influenza vaccine for the season 2005-2006 is an example of mismatch between the composition of the vaccine and the circulating strains. The dominating virus strain circulation in the Norwegian population was B/Malaysia/2506/2004 while the influenza vaccine contained the B/Shanghai/361/2002 virus strain. Lack of effect was therefore observed and WHO recommended that the influenza vaccine for 2006-2007 season should contain an influenza B/Malaysia strain [**4**]. If this virus had been a more virulent one, for example a influenza type A virus, an epidemic would possible have developed.

The WHO recommendation for vaccines to be used in the 2006-2007 season in the northern hemisphere should contain the following viral strains [**5**]:

- an A/New Caledonia/20/99(H1N1)-like virus
- an A/Wisconsin/67/2005 (H3N2)-like virus
- a B/Malaysia/2506/2004-like virus

The first vaccine was prepared in 1936 from inactivated influenza viruses [**1**]. Now, the influenza vaccine is available in three types, as an inactivated whole-virus vaccine, split virus vaccine consisting virus particles disrupted by detergent treatment and a subunit virus vaccine consisting essentially of hemagglutinin and neuraminidase from which other virus components have been removed. The influenza vaccine contains two types of influenza A viruses and one type of influenza B virus strains [**6**].

Unlike the influenza vaccine, antiviral agents are not dependent of the antigenic make-up of the circulating viruses and they are effective in both prophylaxis and treatment. Two classes of influenza antivirals are now available for use, M2 inhibitors and neuraminidase inhibitors, each class inhibits different steps in the viral replication. Antiviral agents may be particularly important for pandemics, especially in the early months, when the supplies of vaccines are likely to be severely limited.

Figure 1 Classification of disease by incidence

The history of influenza epidemics and pandemics can be traced back with some accuracy for the past three hundred years. After the discovery of the influenza virus, outbreaks has been recorded and confirmed by laboratory diagnosis. In the two centuries before this time, infections were identified by the known signs and symptoms of disease and the explosive nature of outbreaks. Originally the term influenza came from the Italian form of Latin *influential*, meaning "epidemic", because they believed that epidemics were though to be due to astrological or other occult influences [**7**].

Epidemics of influenza are characterized by a high number of infected patients, occur suddenly without warning, and disappear after a few weeks or months. In annual epidemics 5-15% of the population is affected. Influenza type A virus leads to epidemic outbreaks almost every year, whereas influenza B virus epidemics are less common. The reason for this is that influenza B viruses lack an animal host reservoir in addition to less frequent antigenic drift. According to historical sources, pandemics have appeared with intervals since ancient times. Historians of medicine agree that 10 pandemics have occurred within the last 300 years and the point of origin for all of them is suggested to be China/Russia/Asia. This indicates that the next pandemic also will emerge from this area and the virulent strain is likely to be the highly pathogenic H5N1. There are several reasons for why this area is likely to be the point of origin. One quarter of the worlds population lives in China and ducks, pigs and humans live very closely together. This increases the risk for reassortment to occur and pandemics to develop [**8**].

Pandemic is a worldwide epidemic. These appear suddenly in a specific geographic area, spread throughout the world infecting millions and cause a large numbers of deaths. A pandemic is caused by a new influenza virus A subtype, the HA of which is not related to the influenza virus circulating immediately before the outbreak, and could therefore not have arisen from those viruses by mutation.

Pandemics occur at 10-50 years intervals, and can affect up to 50 % of the population. According to this, the next pandemic is likely to occur approximately within 40 years of the last, which will be either before 2008 (counting from the pandemic in 1968) or in 2017, depending on whether or not the pandemic in 1977 is accepted [**8**].

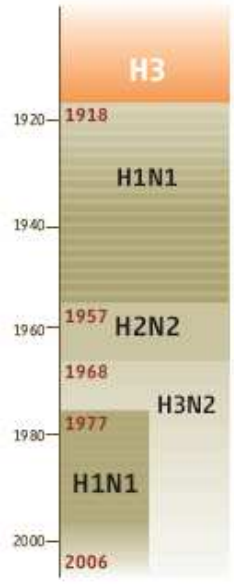


Figure 2 History of influenza [10]

The most severe influenza outbreaks include [**9**]:

- 1918-19 "Spanish flu" A (H1N1)
  >30 million deaths worldwide
- 1957-58 "Asian flu" A (H2N2)
  1 million deaths worldwide
- 1968-69 "Hong-Kong flu" A (H3N2)
  800,000 deaths worldwide
- 1977-78 "Russian flu" A (H1N1)

2.1.1 Influenza versus the common cold

Signs and symptoms	Influenza	Cold
Onset	**Sudden**	**Gradual**
Fever/chill	Common	Rare
Cough	Usual; severe	Less common; mild to moderate
Headache	Prominent	Rare
Muscle aches and pains	Usual and often severe	Slight
Fatigue and weakness	Usual; last up to 2-3 weeks	Very mild

2.2 VIRUSES [11]

Viruses are a major class of microorganisms. They are not dynamic open systems, taking in nutrients or expel wastes. A virus particle is a static structure, quite stable and unable to change or replace its parts. Only when it infects a cell does a virus acquire the key attribute of a living system, reproduction. Unlike cells, viruses have no metabolic abilities of their own. And although they contain their own genes, viruses lack ribosomes and therefore depend on the cell's biosynthetic machinery for protein synthesis. Some viruses do also use the host cell DNA and RNA polymerases for replication and transcription, respectively.

Many viruses causes disease in the organism they infect, but virus infection does not always lead to disease. As an example, avian species can be silent reservoir of influenza A viruses.

A fully assembled infectious virus is called a virion. The simplest virions consist of two basic components: nucleic acid (single- or double-stranded RNA or DNA) and a protein coat (the capsid, which functions as a shell to protect the viral genome from nucleases and which during infection attaches the virion to specific receptors exposed on the prospective host cell). Capsid proteins are coded for by the virus genome. Some virus families also have an additional covering called the envelope. Enveloped viruses enter the host cell by fusing either with the plasma membrane or with the endosomal membrane following endocytosis. Virus envelopes can be considered an additional protective coat. And the fusion of a virus is regulated to ensure that the virus particles only fuse with the appropriate host cell membrane and not to other virus particles.

2.3 INFLUENZA VIRUS

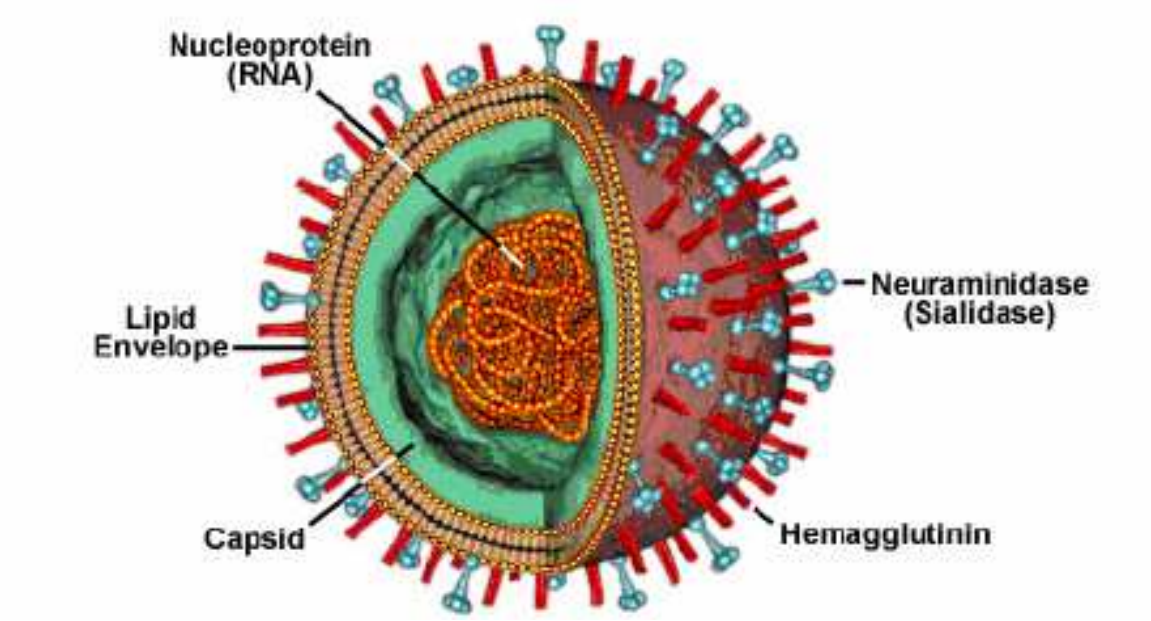


Figure 3 Influenza virus [9].

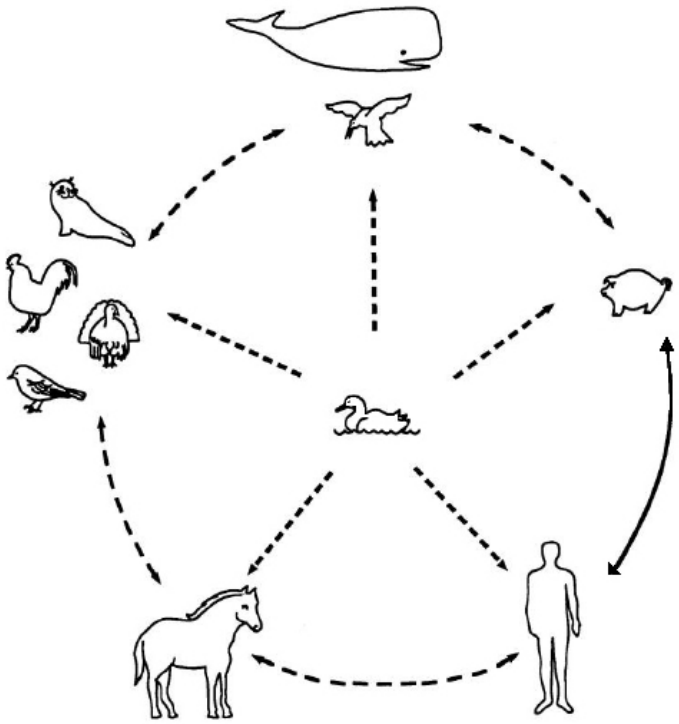
Classification

The influenza viruses belong to the family *Orthomyxoviridae* (from the Greek *orthos*, meaning "standard, correct", and *myxa*, meaning "mucus"). The *orthomyxoviridae* family contains four genera of viruses: influenza A, B and C viruses, and thogotovirus (sometimes called influenza D viruses) [**7**]. Little is known about the thogotovirus and is therefore not discussed any further in this thesis.

The classification of the influenza viruses are based on their antigenic differences between their nucleocapsid and matrix proteins. The genomes of influenza A and B viruses consist of eight segments of negative sense RNA whereas influenza C viruses possess only seven negative-sense RNA segments. Only types A and B viruses cause clinical disease of any concern. The influenza C virus causes less severe respiratory illness which rarely progresses to the lower respiratory tract and is most commonly associated with sporadic illness in children [**12**].

Influenza A viruses are further classified into subtypes based on antigenic differences of the surface proteins hemagglutinin (HA) and neuraminidase (NA), respectively 16 and 9 subtypes

have been identified. Influenza type B viruses are not divided into subtypes. The influenza C viruses lack the gene which is equivalent to the neuraminidase gene of A and B viruses, and has only a single multifunctional glycoprotein, the hemagglutinin-esterase-fusion protein (HEF) [**7**].



The different kinds of influenza viruses also infect different hosts and have different natural habitat. Influenza A viruses are found in animals including pigs, horses, a variety of avian species and humans. In contrast, there is no evidence that influenza B viruses infect other hosts besides humans and seals. Although influenza B virus is mostly associated with lower attack rates and a milder disease, it may occasionally cause epidemics of the same severity as type A viruses[**7**].

Figure 4 The reservoir of influenza A viruses [7]

The currently circulating influenza viruses that cause human disease are of the H1, H2 or H3 and N1 or N2 subtypes and all of the currently identified 16 HA and 9 NA subtypes of influenza A viruses are maintained in wild, aquatic bird populations. Genome segmentation facilitating gene reassortment and high mutation rates are the basis for the extraordinary genetic variability of influenza viruses. Type B viruses do not exhibit the same degree of antigenic variation as does type A [**13, 14, 15**].
Viral isolates are described according to type, geographical origin, strain number, year of isolation and subtype, in this order (e.g. A/Sydney/5/97 (H3N2)).

Virion structure

The O*rthomyxoviridae* are composed of about 1% RNA, 5-8% carbohydrates, 20% lipid and 70% protein [**7**]. They are enveloped viruses that contain a segmented negative single stranded RNA (ssRNA) genome. The genome of these viruses encodes at least 10 proteins. The viruses are pleomorphic, but usually spherical, filamentous and approximately 80-120 nm in diameter. The viral envelope is composed of a lipid bilayer containing transmembrane proteins on the outside and matrix protein on the inside. The lipids are derived from the host plasma membrane but are selectively enriched in cholesterol and glycosphingolipids. Two transmembrane envelope proteins, hemagglutinin and neuraminidase are anchored in the lipid bilayer of the viral envelope. Both of these proteins are essential for viral replication. Influenza type A has also an third transmembrane protein, M2, an ion channel [**16, 17**]. These proteins are target for antiviral treatment.
Influenza A viruses most striking feature is the layer with about 500 spikes (NA and HA) radiating outward. Usually the viruses have 4-5 times more HA spikes than NA.

2.3.1 Influenza virus replication cycle

The life cycle of influenza virus involves attachment to cell surface receptors, entry into the cell and uncoating of the viral RNA followed by replication of the viral genes inside the cell nucleus. After the synthesis of new copies of viral proteins and genes, these components assemble into progeny virus particles, which then exit the cell by budding from the cell surface. The replication site for influenza viruses are the epithelial cells of the upper respiratory tract of humans, horses, and pigs. They replicate extremely well in humans and one replication cycle takes only 4-6 h and kills the host [**18**].

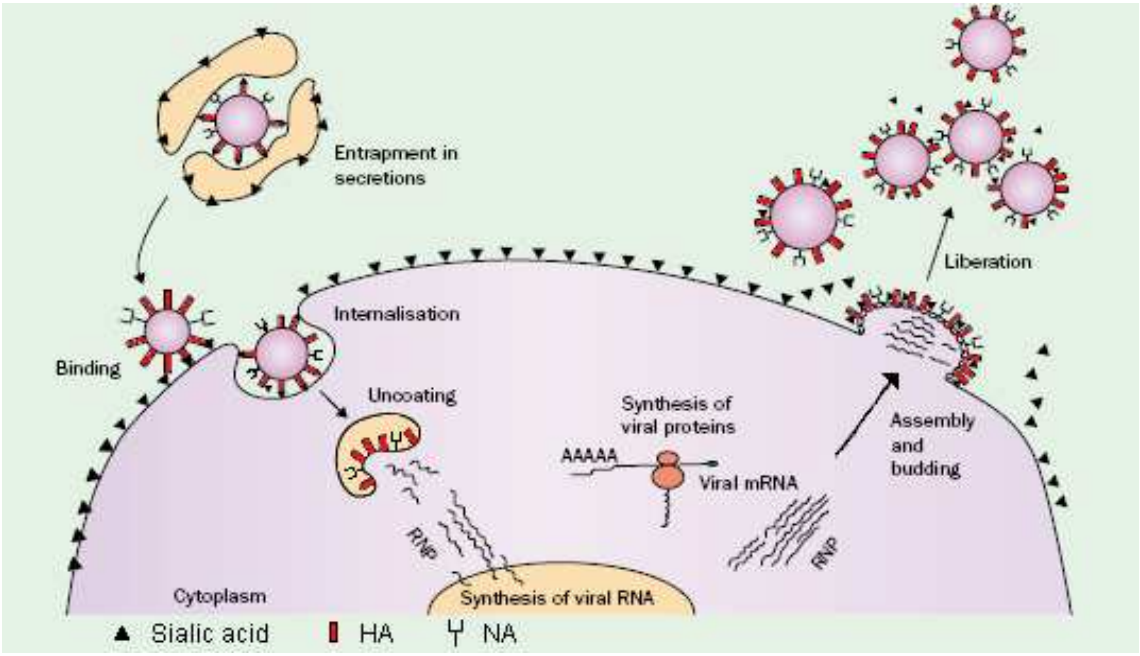


Figure 5 Influenza virus replication cycle [19].

The HA glycoprotein is responsible for the binding of influenza viruses to their host cells. The HA interact with receptor molecules containing sialic acid on the cell surface, initiating virus penetration and promotes fusion between the viral envelope and the cell membrane.
The influenza virus penetrates into the cell by receptor mediated endocytosis.
After the entry into the cell, the low pH in the endosome induces an irreversible conformational change in HA, which ultimately result in membrane fusion between the viral and cellular membranes. The acidification of endosomes is brought about by H^+-ATPase and activates viral M2 ion channels which allow protons to enter the interior of the virus particle. The ribonucleoprotein segments (RNP) are released from the endosome when the endosomal pH is decreased to ~ 5.0. Viral RNA strands are replicated in the nucleus and new virus particles are produced.

The main role of NA is the release of newly manufactured virions from the cell. NA destroys receptors recognized by HA by cleavage of the terminal sialic acid residue from carbohydrate moieties on surface of host cells. This cleavage facilitates movement of the newly formed virus from the surface of infected cells.

2.3.2 Neuraminidase (NA)

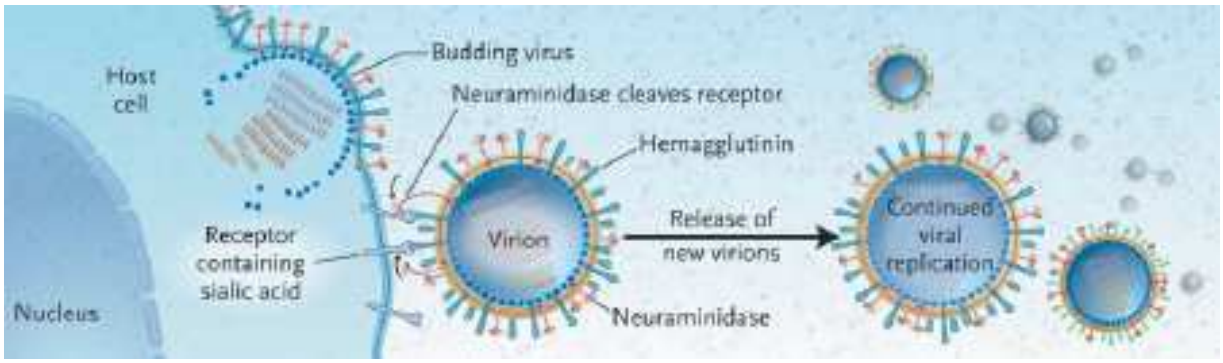


Figure 6 Neuraminidase activity [3]

Neuraminidase (NA) is an integral membrane protein and one of the two subtype specific glycoprotein of influenza A and B viruses. NA is an enzyme, a glycohydrolase/sialidase that cleaves terminal α-ketosidically linked sialic acids from a large array of glycoproteins, glycolipids and oligosaccharides [**20**]. NA is found on the surface of influenza virus and is a homotetramer which accounts for about 5-10 % of influenza virus protein (Figure 3 above). NA forms a mushroom-shaped spike with a box shaped head, which is enzymatically active, and has a centrally attached stalk containing a hydrophobic region by which NA is embedded in the viral membrane. The NA is a prototype class II integral membrane protein, with the N-terminal in the cytoplasm (as anchor) [**21**].

By catalyzing the cleavage of sialic acid residues on the surface of infected cells the enzyme:

- promotes the release of virion
- prevents the formation of viral aggregates after release from host cells
- may prevent viral inactivation by respiratory tract mucous and promote the spread of virus in the respiratory system
- may contribute to viral pathogenicity
- may induce cellular apoptosis and release of pro-inflammatory cytokines [**22**].

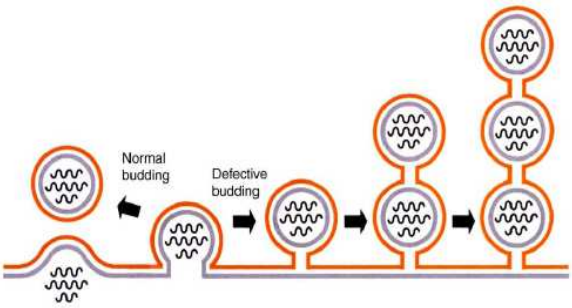


Figure 7 Influenza virus budding [16]

NA performs a vital function in the final stage of the viral life cycle. Without functional NA, newly formed viral particles remain attached to the host cell membrane and form viral aggregates.

Nine subtypes of neuraminidase, N1-N9, have been identified for influenza virus type A. No subtypes of NA have been isolated for influenza type B. Difference between the type A and type B NA amino acid sequence is close to 80%, but the amino acids that bind the glycosidically linked sialic acid or its transition state are totally conserved in spatial arrangement as well as in chemistry [**23**].
Despite the considerable diversity of the sequences, the residues lining the active site of NA are conserved in all wild-type influenza viruses. This makes the active site of NA an excellent target for a broad spectrum inhibitor [**24**]. There are 11 amino acid side chains directly in contact with the substrate that never vary in the active site of NA (Figure 14 below) [**23**].

Organisms that contain neuraminidase include bacteria (*Vibrio cholerae, Clostridium perfringens, Streptococcus pneumoniae and Arthrobacter sialophilus*) and viruses (especially *orthomyxoviruses* or *paramyxoviruses* such as influenza virus A and B, *parainfluenza virus, mumps virus, Newcastle disease virus, fowl plague virus* and *sendai virus*) [**25**].

The neuraminidase is also thought to act on the mucin layer in the respiratory tract. Removal of the sialic acid lower the viscosity and produce liquefaction, which together with mucociliary transport, may help spread the virus through the respiratory tract and permit access to the epithelial cells [**26**].

The presence of NA on the surface of virions seems contrary to the requirements for viral attachment to cells. But different studies of NAs biological role have observed that when a virus was culturated in the presence of an neuraminidase inhibitor (NAI), it was restricted to a single replication cycle because the progeny became immobilized at the surface of the

infected cell (Figure 7) [**27**]. The NA does therefore not interfere with the entry process of the virus.

2.3.3 Hemagglutinin (HA)

The name of hemagglutinin has originated from the influenza viruses ability to agglutinate erythrocytes by attaching to specific sialic acid containing receptors [**7**]. The hemagglutinin (HA) spike appears to be rod shaped and protrudes from the envelope as a trimer. HA is a type I glycoprotein, containing an N-terminal ectodomain and a C-terminal anchor. There has been identified 16 subtypes of HA, H1-H16 [**28**]. And the HA are found on the surface of the influenza virus (Figure 3 above).

The HA facilitates entry of the virus by binding to sialic acid containing receptors on the cells surface, the initial step in virus replication [**21**]. HA is also responsible for penetration of the virus into the cell cytoplasm. By mediating the fusion of the membrane of the endocytosed virus particle with endosomal membrane HA causes release of the viral nucleocapsids into the cell cytoplasm [**7**].

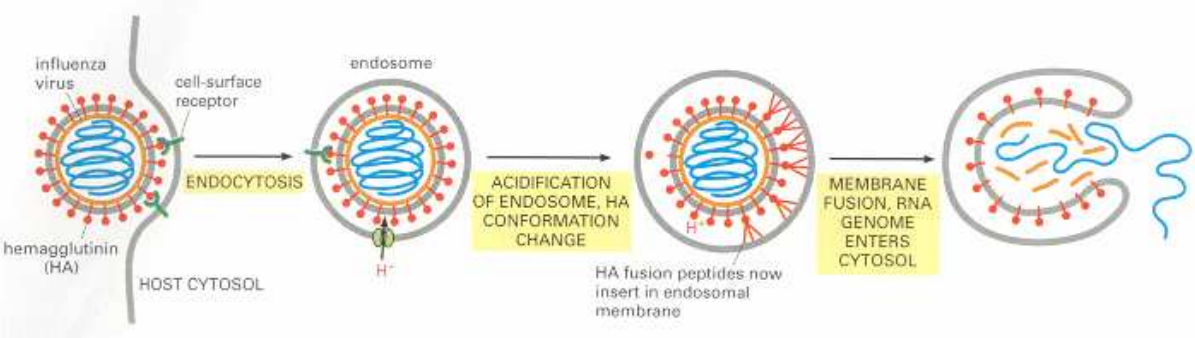


Figure 8 The entry strategy used by influenza virus [11]

HA represents the major antigenic determinant of influenza types A and B, and induces neutralizing antibodies. For survival, influenza viruses must evade immune recognition by process of continual evolution.

The HA can exist in two different forms, depending on the virus strain, host cell type and growth condition. The HA precursor (HA_0) is either cleaved protolytically into two disulfide-linked chains, HA_1 and HA_2 or remains uncleaved. The HA_1 subunit carries the sialic acid binding site, and the HA_2 subunit is responsible for fusion of viral and cellular membranes. Uncleaved HA cannot undergo the low pH induced refolding events associated with HA mediated membrane fusion (Figure 8) [**7, 29**].

2.3.4 M2 protein

The M_2 protein of influenza A virus is the prototype viral ion channel protein. It is an small integral membrane protein (type III) that is abundantly expressed at the plasma membrane of influenza virus infected cells, but only a small amount is incorporated into budding virions [**30, 31**].

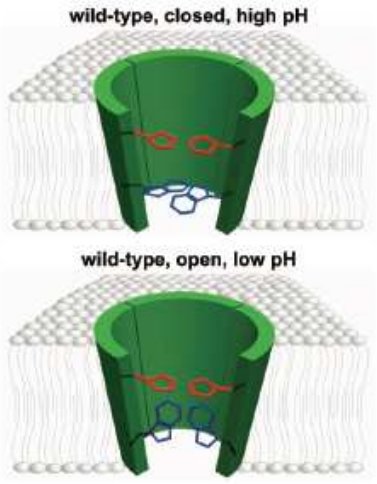


Figure 9 Model for activation of the M_2 ion channel [31] The model is showing only the transmembran domain from residues 24 to 44 (green), His^{37} (red) and the gate Trp^{41} (blue). And these residues are shown for only two of the four subunits.

The M_2 protein has a high selectivity for protons (H^+). Even though the M_2 protein is a minor component of the viral envelope, the ion channel activity is nonetheless essential in the life cycle of the virus. After the influenza virus has entered the cell by endocytosis, the low pH in the endosomal compartment activates the M_2 ion channel. The acidifying of the interior is essential for the uncoating of virions (Figure 8 above). The M_2 protein is gated by pH, and this property is mediated by the indole moiety of Trp^{41}.When the pH outside the endosome is high, the indole moiety of the side chain of Trp^{41} is blocking the pore of the M_2-channel. With low pH outside the His^{37} is charged, allowing rotation of Trp^{41} to a conformation parallel to the pore`s axis, permitting H^+ to flow (Figure 9) [**31**].

The influenza B virus lacks the M_2 proteins as found in influenza A viruses. Instead influenza B viruses expresses a BM_2 protein with is thought to have ion channel activity [**7**].

2.3.5 Antigenic drift and shift

Genes of influenza viruses mutate with high frequency, particularly the HA and NA glycoproteins against which immunity is directed. Serum antibody to the virus HA is the most important factor in immunity. The accumulation of point mutations in the HA and NA genes may lead to gradual antigenic change of surface glycoproteins (antigenic drift) and the emergence of immunologically distinct strains (antigenic shift). Both of the surface antigens of influenza virus undergo extensive variation. Influenza A viruses undergoes frequent changes in their surface antigens, whereas type B influenza viruses change less frequently [**15**]. The segmented genome of influenza virus facilitates the exchange of genetic information between different influenza viruses (reassortment). This eight different segments can theoretically produce 256 different combinations of RNA from the shuffling of the virus [**7**].

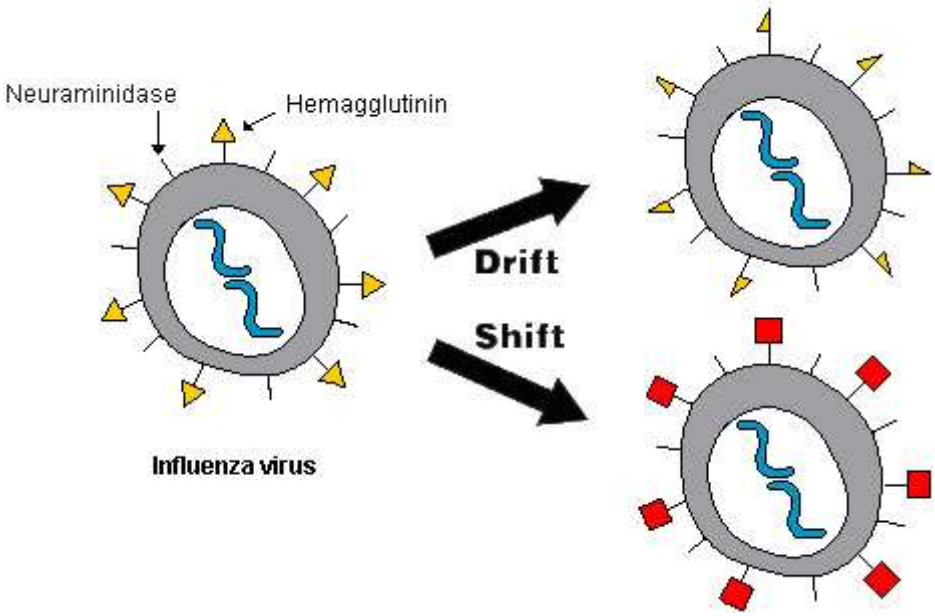


Figure 10 Antigenic drift and shift [32]

2.3.5.1 Antigenic drift

Drifts are the natural mutation over time of known strains of influenza to evade the immune system. Because RNA-directed RNA replication has no quality control mechanism, in contrast to DNA replication, RNA viruses are generally more susceptible to the generation of mutation leading to serotype formation than the DNA viruses. Errors are produced in $1/10^4$ bases per replication cycle for RNA-dependent RNA polymerase, whereas DNA polymerase produces replication errors only in $1/10^9$ bases per replication cycle [**33**]. This point mutations

leads to amino acid sequence changes in the antibody binding sites (epitopes) on these virus proteins. The epitopes of the present virus variant is no longer recognized by human antibodies, thereby permitting the virus to multiply and capable of causing illness through evasion of the immune response [**2**]. Antigenic drift occurs in all types of influenza including influenza A, B and C. Antigenic drift has also been detected among avian influenza viruses, but to a lesser extent than in human viruses. The reason for this is that birds are short lived and therefore have limited immunological pressure [**2, 21, 34**].

2.3.5.2 Antigenic shift

Antigenic shifts are far less frequent and are the process by which two different strains of influenza viruses combine to form a new subtype having a mixture of the surface antigens of the two original strains. This event occurs when two different viruses, possibly each from a different host species, co-infect a single host, for instance a pig. The pig then acts like a mixing vessel and the genome segments of the two viruses can undergo reassortment. The pathogenicity of the new virus are unpredictable, it may lack the requisite virulence factors (and therefore get attenuated or frankly non-viable) or it may possess full virulence for humans, plus a new surface antigen, usually the HA (from an animal host virus). Such a new virus has pandemic potential because it may be intrinsically pathogenic in humans and have surface antigens against which the human population lacks any significant immunity. Once a pandemic strain is created, it may change its virulence further as it continues to replicate, adapting to the host as it does so. Antigenic shift occurs only in influenza A because it infects more than just humans. Influenza type B does not exhibit antigenic shifts and therefore do not have pandemic potential. This is because influenza B viruses lack an animal reservoir and has only one HA and NA subtype [**2, 32, 34**]

2.4 TREATMENT OF INFLUENZA

The most important protective response against invading pathogens is immunological and immunity after an influenza infection may last for many years. For many other viruses, a single infection of a young child confers a lifelong immunity.

The immune system produces both interferons and antibodies under the infection of influenza virus. The interferons are produced shortly after the viral titer has reached its peak, while

antibodies (IgG is most important), are not detected until later in the recovery and are responsible for the final clearing of the virus [**26**].
Inactivated influenza virus vaccines have been used in prevention of influenza viruses for about 50 years. Although vaccination has been successful in eliminating a few viruses from the world (e.g. smallpox and polio), many viruses have developed ways to escape from the immune system. The antigenic variation of influenza viruses was detected in the 1940s. The influenza virus has a limit to how much variation it can tolerate. Mutation to escape neutralizing antibodies of the immune system must also preserve the function of the surface proteins. An ideal influenza vaccine would be directed against these essential regions, where mutation would result in loss of viability. Crystal studies of the surface glycoproteins shows that these regions usually are in depression on the viral surface and are generally inaccessible to antibodies. They are however accessible to small molecules and this is the reason for why so much effort is being directed toward the development of antiviral drugs that bind to these conserved sites and inhibit the viral function [**23**].

Until a few years ago, there were only two options available to reduce the impact of the influenza virus, vaccines and the antiviral M2 inhibitors, amantadine and rimantadine. Although protection through vaccination is limited due to the frequent antigenic shift, vaccination has for a long time been, and still are, the first line defence against influenza virus [**35**]. The use of M2 inhibitors is now limited, because of rapid emergence of resistant viral strains which reduces their effectiveness, its relatively high frequency of adverse effect and lack of activity against influenza B virus. In 1999 the first neuraminidase inhibitor was approved for treatment of influenza. Zanamivir (Relenza®), developed by GlaxoSmithKline, was the first drug in this new class of inhibitors and designed after crystal studies of the neuraminidase enzymes active binding site. Three years later Roche licensed the second neuraminidase inhibitor, oseltamivir (Tamiflu®). Unlike zanamivir, oseltamivir was approved for both prophylaxis and treatment of influenza virus. This class of inhibitors has a major advantage, they target a highly conserved region in the neuraminidase enzyme and resistance is therefore less likely to occur. Oseltamivir is now the first choice of the antiviral drug against influenza virus and is effective against all strains of influenza virus, also the threatening and potential pandemic H5N1 strain.

2.5 ANTIVIRAL AGENTS AGAINST INFLUENZA VIRUS

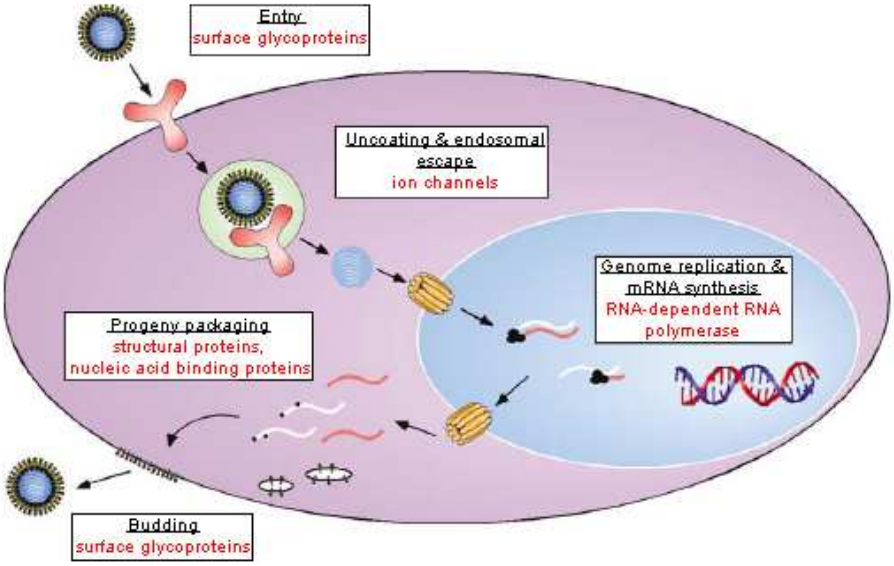


Figure 11 Potential antiviral targets in the influenza virus (modified from Leonard *et al.* [36])

The basic biology and reproductive mechanism varies widely between different viruses and unique strategies are needed to inhibit each particular virus. In the figure above, some of the potential viral targets in the influenza virus are displayed. Currently only antiviral drugs which inhibit the uncoating/endosomal escape and the budding process are approved for medical use (Table 1). Small interfering RNAs (siRNAs) which interfere the replication of mRNA and inhibitors of influenza virus polymerase are studied as potential antiviral agents, but much work remains to be done before clinical success is realized [**36, 37**]. The structure of the compounds under clinical and preclinical evaluation can be found in Figure 15 below.

Table 1 The past, present and future of antiviral compounds against influenza virus[1]

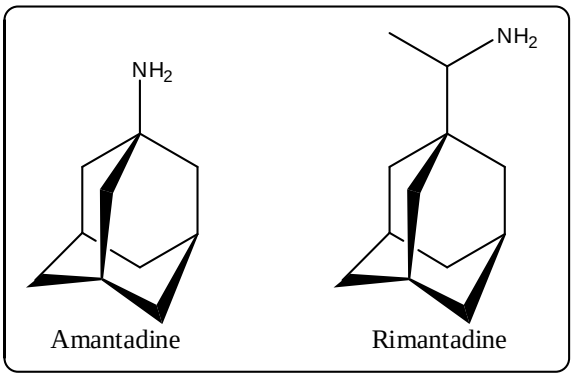
			Compound		
VIRUS	**Viral target**	**Comments**	Approved for medical use	In clinical development	In preclinical evaluation
Orthomyxo	Matrix (M_2) protein	Target for inhibition	Amantadine Rimantadine		
	Neuraminidase	Target for inhibition	Zanamivir Oseltamivir	Peramivir [**39**]	A-192558 A-315675

[1] Modified from reference 38. E. E. De Clearcq, *A guided tour through the antiviral drug field*, Future Virology 1 (2006), no. 1, 19-35.

There are two classes of antiviral agents against influenza viruses, the M_2 inhibitors and the neuraminidase inhibitors. These two classes of antivirals are effective against different types of influenza viruses.

Even though hemagglutinin plays a major role in the influenza viruses entry process of the cell, blocking of hemagglutinins sialic binding site may not be the best strategy for designing antiviral agents against influenza viruses. This is because studies have indicated that influenza viruses can enter cells without the assistance of sialic acid and also in the absence of a functional sialic acid binding site on hemagglutinin [**23**].

2.5.1 M2 inhibitors, the amantadanes



The amantadanes were identified by traditional biologic screening assays in the early 1960s [**40**]. Amantadine and its analogue rimantadine were the first antiinfluenza drugs and block the ion channel of virus protein M_2 (Figure 9 above). These drugs block the acidification of the virions interior and the release of transcriptionally active ribonucleoprotein complex for the transport to the nucleus. A second additional effect of amantadine and rimantadine is the blockade of HA maturation during the transport from the endoplasmic reticulum to the plasma membrane [**7**].

The M_2 inhibitors are only active against type A influenza viruses, because the influenza B virusese lack the M_2 protein [**41**]. Amantadine and rimantadine were approved for the prevention and treatment of influenza A in 1976 and 1993, respectively [**42**]. However, these drugs are not available on the Norwegian marked and they have never achieved widespread acceptance because of the rapid development of viral resistance, their lack of activity against influenza B and, in the case of amantadine, adverse events (e.g. anxiety, lightheadedness, seizures) [**7**]. These adverse events are also more evident in elderly patients.

Rimantadine exhibit fewer side effects in the central nervous system than amantadine at comparable doses, the reason for this might be because amantadine is excreted intact in the urine, while >90 % of the rimantadine dose is metabolized through hydroxylation at multiple sites [**43**].

Complete cross-resistance against amantadine and rimantadine is associated with a single nucleotide change in the M_2 protein after a single passage *in vitro* and from a single host *in vivo* [**23**]. The resistant variants are also transmissible and fully pathogenic.

Amantadine has also an anti-parkinson effect which was serendipitously discovered during a course of prophylaxis for influenza [**40**]. And amantadine are now recommended for use in elderly patients with drug induced parkinsonism [**44**].

2.5.2 Neuraminidase inhibitors (NAI)

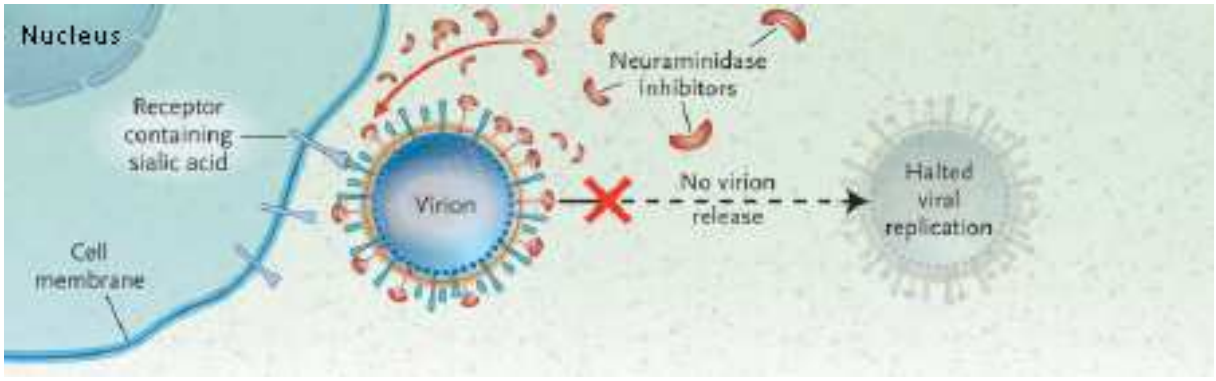


Figure 12 Neuraminidase inhibitors [3]

The neuraminidase inhibitors (NAI) interfere with the release of progeny influenza virus from infected host cells. Thereby it prevents infection of new host cells and halts the spread of the infection in the respiratory tract. NAIs are effective against both influenza type A and B, and the inhibition is reversible [**45**].

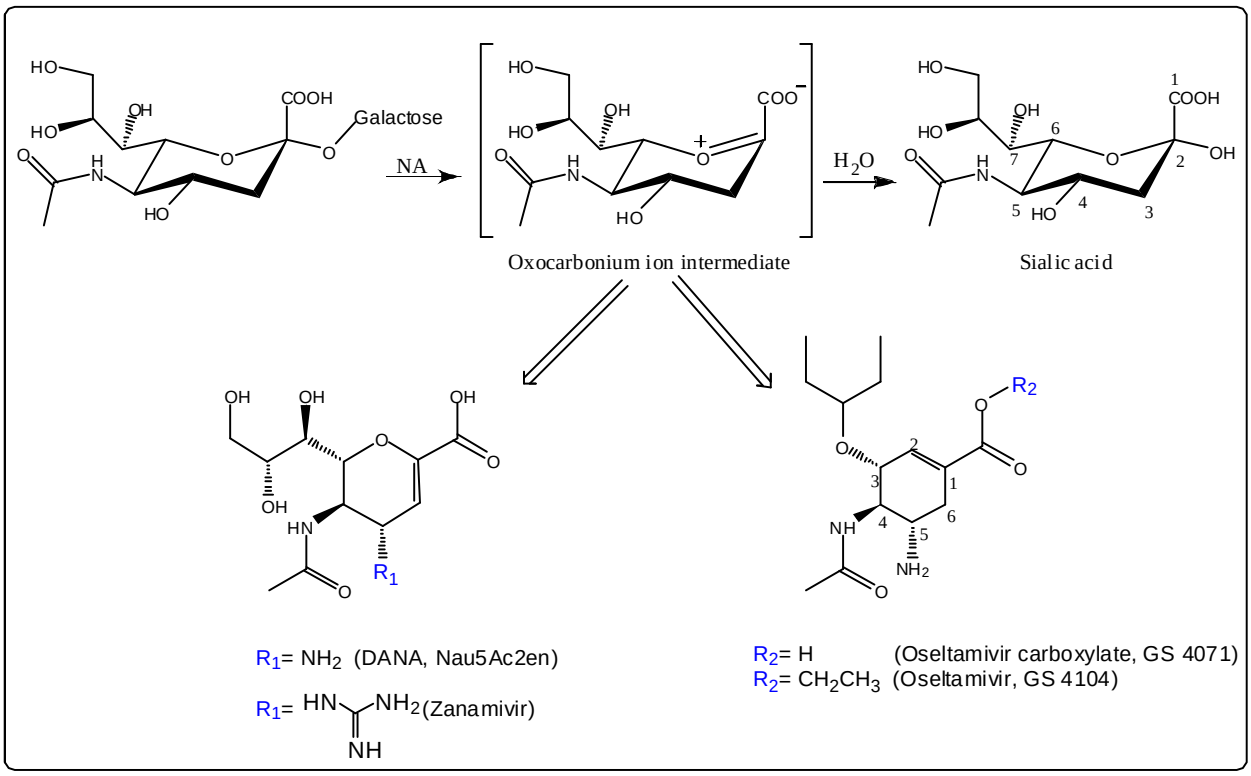


Figure 13 Mechanism of neuraminidase hydrolysis [46]

Two neuraminidase inhibitors are currently licensed as drugs for the treatment of influenza virus, they were both invented after determination of the crystal structure of the influenza virus NA in 1983. They are developed by structure-based drug design, to target conserved residues at the neuraminidase enzyme active site in influenza A and B viruses [**23**].
The structure clarification also led to the recognition of the oxocarbonium ion as the likely intermediate in NA hydrolysis, and hence analogs were synthesized as likely candidates for inhibitors (Figure 13) [**23**]. The first inhibitor to be synthesized was DANA (2-deoxy-2,3-dihydro-N-acetylneuraminic acid, Neu5Ac2en), which were effective *in vitro* but did not inhibit replication of influenza viruses in animals. After the structure clarification of NA, DANA served as the lead compound in the rational design of drugs targeting the NA.

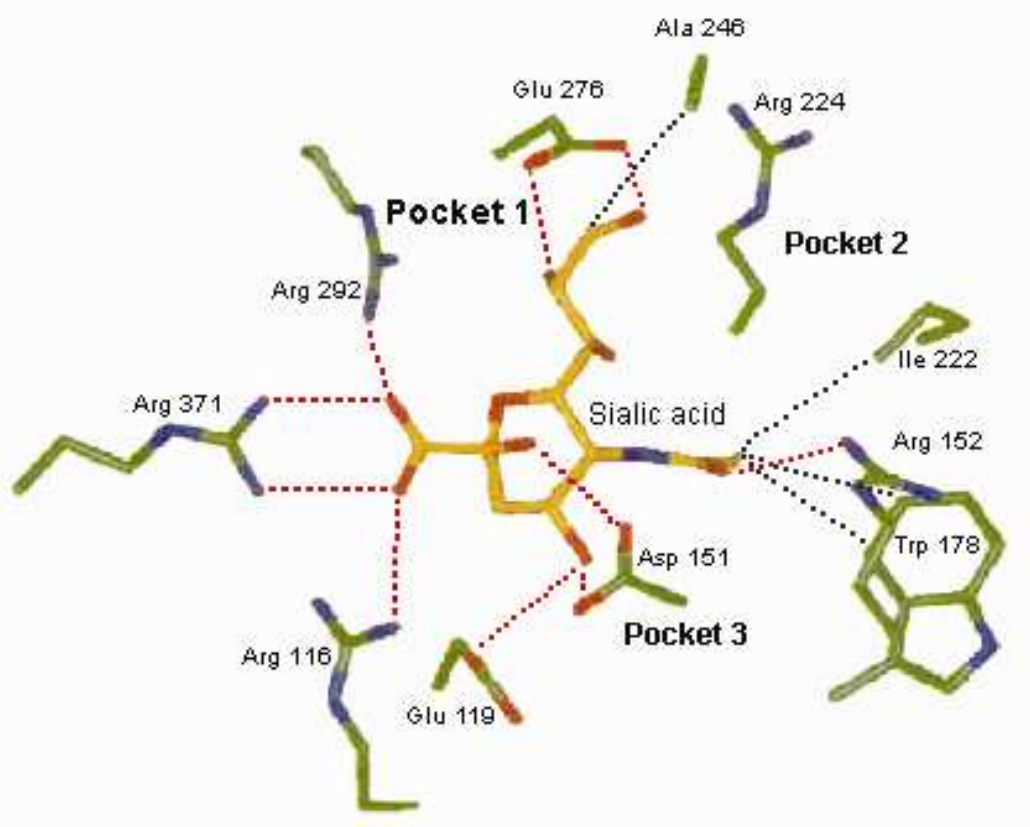


Figure 14 Sialic acid in the active site of neuraminidase [35]

Atoms are colored as following: red for oxygen, blue for nitrogen, green for NA carbon and orange for sialic acid carbon. Dashes indicate H-bonding (red) and any hydrophobic (black) interactions, only side chains are shown and some active residues are omitted for clarity.

2.5.2.1 Zanamivir (Relenza®, GlaxoSmithKline)

Zanamivir (Figure 13 above) is a sialic acid analog which was first described in 1993 by von Itzstein *et al.* [**20**]. Zanamivir was designed after study of DANAs binding to neuraminidase (DANA is the unsaturated sialic acid analogue). Zanamivir (Relenza®) was the first inhibitor to be synthesized which specific inhibited the neuraminidase and was approved for influenza treatment in 1999. Zanamivir is modified at C4, where hydroxyl group of sialic acid is substituted with a guanidine group [**47**]. This replacement filled an unoccupied pocket in the NA active site and increased the binding interactions, which was confirmed by x-ray crystallography [**7**].

Zanamivir is a highly polare molecule, which does not penetrate cell membranes readily. This confers a low bioavailability and zanamivir must therefore be administered by inhalation.

Zanamivir is contraindicated in patients with underlying respiratory disease because of the risk of serious adverse events like bronkospasm and allergic-like reactions might occur.

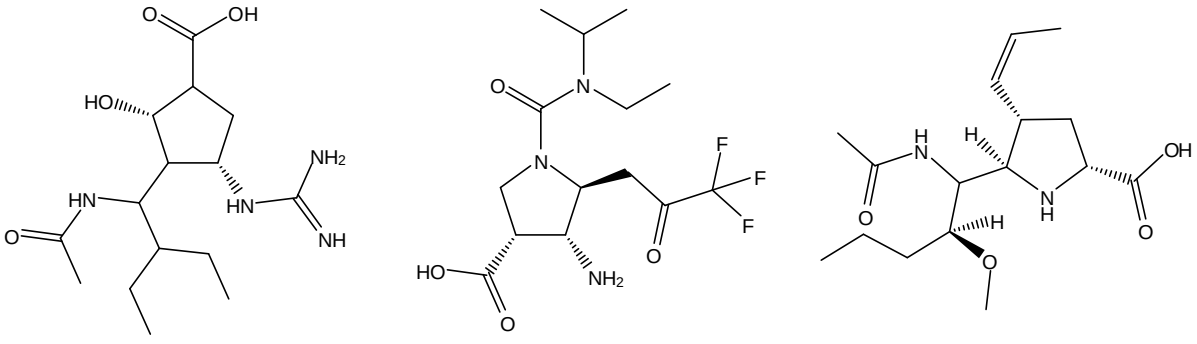
2.5.2.2 Oseltamivir (Tamiflu®, Roche)

Oseltamivir is the ethyl ester prodrug of the neuraminidase inhibitor oseltamivir carboxylate. Oseltamivir carboxylate has a cyclohexene ring instead of a sugar ring and modifications at both C4 (hydroxyl group → amino group) and C6 (glycerol side chain → pentyl ether group), compared to the oxocarbonium ion intermediate [**47**]. When given as an early treatment of influenza oseltamivir shorten the duration of illness by 1.5 days.

View point 2.7 TAMIFLU® (ROCHE) below.

2.5.3 Other neuraminidase inhibitors

Screening procedures have identified other molecules that inhibit influenza NA. Plant flavonoides have for example been investigated for years, but they are not very potent (K_i 10^{-5} M) and their mechanism of action is still unclear [**23**].



Peramivir
RWJ-270201
BCX-112

A-192558

A-315675

Figure 15 Potential neuraminidase inhibitors

Figure 15 shows three promising potential neuraminidase inhibitors in different phases in the research and development pipeline. A-192558 and A-315675 are under preclinical evaluation while peramivir has undergone phase III clinical trials. Peramivir has been reported with high NA inhibitory activity and was designed from studies of DANA and its interactions with

influenza neuraminidase. It is a very promising drug and the *in vitro* potency appears to be greater than either zanamivir or oseltamivir carboxylate, based on the generally lower EC_{50} values [**48**].

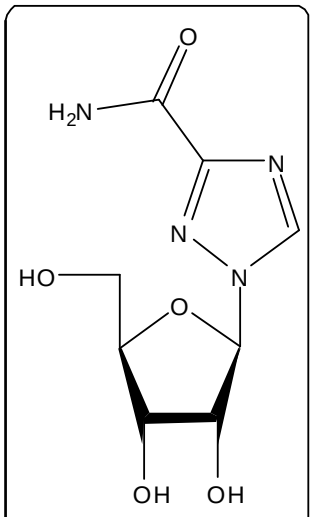
2.5.4 Other antiviral agents against influenza viruses

2.5.4.1 Protease inhibitors

Influenza virus encodes a multifunctional enzyme, RNA polymerase, which catalyzes both transcription and replication of the RNA genome. Although the enzyme is known to be important for the replication, the exact function of the enzyme is poorly understood [**49**]. Most of the marketed antivirals are nucleoside analogue that have been modified to inhibit only polymerases in virus effected cell, but there is still not marketed any specific inhibitor of influenza polymerase. Influenza viruses also uses a protease to cleave the mature form of hemagglutinin (HA_0) to HA_1 and HA_2 [**28**]. But this cleavage is mediated by cellular proteases, so for influenza, this step is no viral target for inhibition [**23**]. Nucleoside analogues have also an major drawback, they are usually toxic compounds.

Ribavirin

Ribavirin is a synthetic nucleoside and is currently licensed in Norway as Copegus® (Roche) and Rebetol® (Schering-Plough). Both of theses drugs are approved for use against chronic hepatitis C, but not as monotherapy. In addition Copegus® can be used in combination therapy in patients coinfected with clinical stabile human immunodeficiency virus (HIV). Ribavirin is not approved for treatment of influenza in Norway, but is used in some countries.



Ribavirin

In vitro studies of ribavirin aerosol has shown inhibitory effect on the influenza A and B viruses and are therefore under preclinical evaluation [**50**]. Ribavirin is a broad spectrum, virustatic chemotherapeutic agent. It is effective against a very wide range of viruses, both RNA and DNA, but the precise mechanism of action is still unknown.

2.5.4.2 Recombinant interferon α

Interferon (IFN) α is a familiy of approximately 20 proteins with similar activities. IFNα plays a normal part of the immunological response to presence of viruses [**51**]. IFNα has been shown to induce B-lymphocytes to differentiate into antibody producing plasma cells and is necessary for the production of IgG in the response of influenza infection. IFN α is a powerful adjunct when mixed with influenza vaccine [**52**].

2.6 RESISTANCE

2.6.1 Resistance to neuraminidase inhibitors

Resistance to neuraminidase inhibitors (NAI) is a major concern in the treatment of a pandemic influenza and WHO established in 1999 the global Neuraminidase Inhibitor Susceptibility Network (NISN). Currently, NISN has not found any influenza isolates with spontaneous resistance.

Resistance to NAI might arise in two ways [**53, 54**]:

1. Mutation of the viral neuraminidase in order to prevent the drug binding to its target. However, NAIs bind to the highly conserved active site of the neuraminidase, this is expected to make the resistance selection difficult and unlikely to develop because they would result in an unfit virus
2. Mutation of the viral hemagglutinin in order to reduce its binding affinity to sialyl residues. This would facilitate viral release without the necessity for neuraminidase activity and, hence, produce virus resistant to NAIs (as a class). The fact that the hemagglutinin mutants have so far been found to be as susceptible to the NAIs as the wild-type viruses in animal experiments, suggests that the neuraminidase may play some vital role other than receptor destruction in the infection process. Possibly the enzyme is required to facilitate the movement of virus particles through respiratory secretions, and thus if it is blocked, the virus may be trapped and immobilized.

In summary, although the development of resistance to the neuraminidase inhibitors in the clinical settings requires further study, available data suggest that resistance to this class of compounds is unlikely to limit their clinical usefulness. This is also supported by the fact that mutant viruses resistant to either zanamivir or oseltamivir carboxylate have been difficult to generate *in vitro* and it is therefore suggested that they might not develop easily *in vivo* [**53**].

However, resistance to neuraminidase inhibitors have been observed in both treated children and adults. But no transmission of oseltamivir-resistant virus between humans have so far been documented [**3**].

2.7 TAMIFLU® (ROCHE)

2.7.1 Description

Tamiflu® (oseltamivir phosphate) is available as a capsule containing 75 mg oseltamivir for oral use, and as a powder for oral suspention (12 mg/ml), produced by Roche.
Tamiflu is indicated for the prophylaxis and treatment of influenza in patients 1 year and older [**55**].
Drug administration guidelines for adults are 75 mg in twice daily doses.

Figure 16 Tamiflu capsules and mixture

2.7.2 Discovery of oseltamivir carboxylate

Oseltamivir carboxylate (GS 4071) was first described by Kim *et al.* in 1997 [**56**].
Oseltamivir carboxylate is a carbocyclic transition state analogue inhibitor of influenza neuraminidase (NA) that was synthesized by researches at Gilead Sciences in California, based on the known structure of the active site of NA.
Oseltamivir was approved for propylaxis and treatment of influenza in Norway in 2002.

Oseltamivir carboxylate is a more potent inhibitor of neuraminidase than zanamivir (K_i 0.3-0.8 versus 1.1-3.5 nM respectively) [**57**].

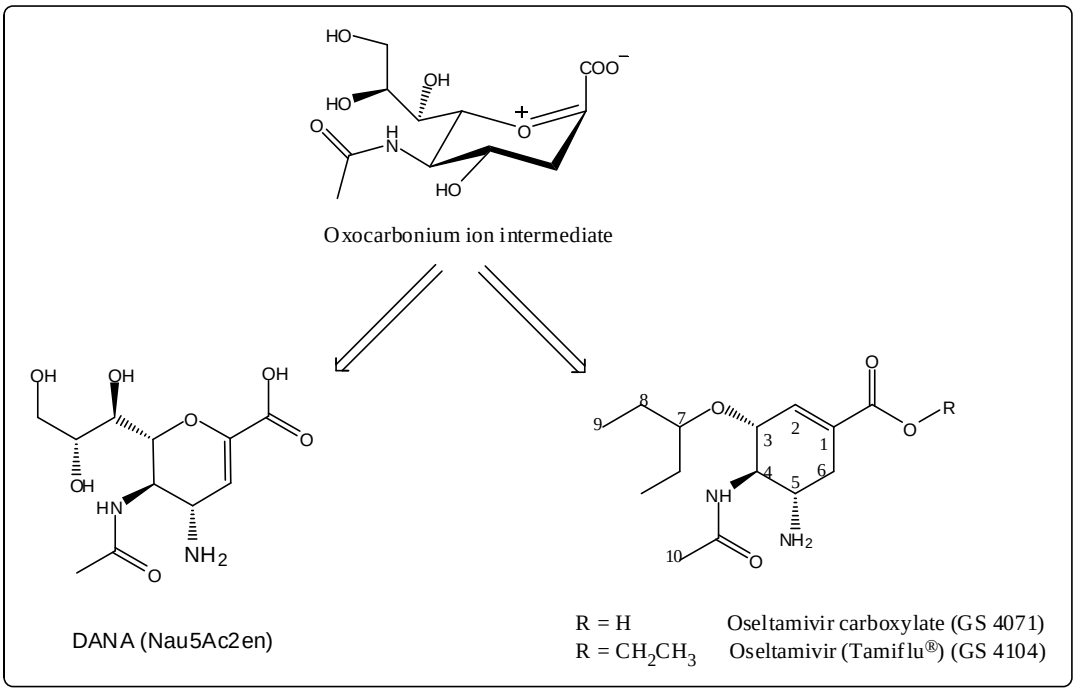


Figure 17 Oseltamivir and oseltamivir carboxylate are designed from DANA

After detailed analysis of the X-ray crystal structure of neuraminidase and the oxocarboniumion intermediate of sialic acid, indications of several critical interactions was detected. Two of these interactions help to anchor the schaffold of sialic acid in the active site of neuraminidase and these interactions are preserved in both oseltamivir and zanamivir:

1) Negative charged carboxylate group which interacts with the positively charged side chains of the three Arg (Arg118, Arg292 and particular Arg 371)

2) N-acetyl group makes both nonpolar and polar contacts with Arg152, Trp178 and Ile222

Figure 18 below shows the interactions between neuraminidase and oseltamivir carboxylate. This figure displays that the NA active site can be divided into three major binding pockets. Pocket 1 is formed by highly polar residues (Glu276, Glu 277, Arg292 and Asn 294) in addition to the hydrophobic Ala 246. Different studies has discovered that although this pocket appears to be highly polar in nature, it has been the key in achieving high binding affinity with cyclohexene based neuraminidase inhibitors [**56**]. The Glu276 side chain can also adopt two different conformations, one of them makes pocket 1 larger and creates a much less polar environment. Pocket 2 is a hydrophobic pocket which is not utilized by sialic acid for binding (Figure 14 above). The third binding pocket is large and contains both hydrophobic and hydrophilic residues.

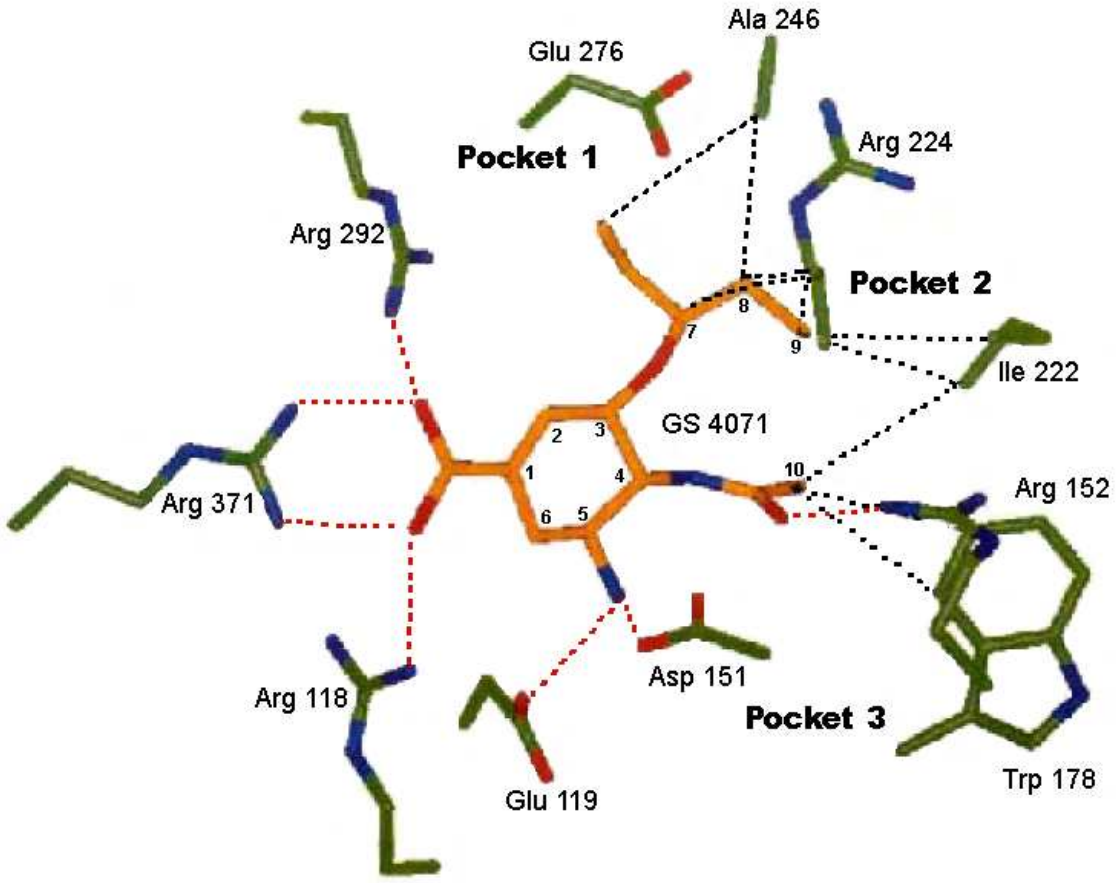


Figure 18 Oseltamivir carboxylate (GS 4071) and neuraminidase X-ray crystal structure [35]
Atoms are colored as following: red for oxygen, blue for nitrogen, green for NA carbon and orange for GS 4071 carbon. Dashes indicate H-bonding (red) and any hydrophobic (black) interactions, only side chains are shown and some active residues are omitted for clarity. Glu276 is also rotated away from the center of pocket 1.

The design of oseltamivir carboxylate is based on that there are no direct interaction between the sugar ring DANA and NA, implying that the position of atoms in the ring is not critical [**23**]. The C-7 hydroxyl of the glycerol side chain (Figure 14 above) did not interact with amino acid residues in the NA active site and therefore an oxygen atom was incorporated as a replacement for the C-7 hydroxy methylene unit. A lipophilic alkyl group was added to this oxygen atom to balance for the effects of the polar functional groups present in the molecule, to enhance the overall oral bioavailability [**35**]. The length, substitution and geometry of this C-3 was determined through several studies of neuraminidase inhibitory activity. These studies indicated that the 3-pentyl group adjacent to the ether oxygen led to a significant increase in potency. This 3-pentyl side chain revealed hydrophobic interaction previously not observed in the sialic acid/neuraminidase complex, resulting in significant increased binding affinity.

The position of the double bound on the neuraminidase inhibitor oseltamivir carboxylate was chosen after several structure activity relationship studies by Kim *et al*. [**56**].

In general, the common characters of importance for binding to NA can be described as followed (numbering from Figure 18 above):

1) A cyclic schaffold connected to three or four substituents – binds in pocket 1
2) A carboxylic group at C-1 position, necessary for strong electrostatic interactions with a triad of arginine residues
3) Two hydrophobic groups at C-3 and C-4 position – binds in pocket 2

Oseltamivir carboxylate is a transition state inhibitor of viral neuraminidase and has demonstrated potent *in vitro* and *in vivo* antiviral activity against influenza virus. However, the oral bioavailability of oseltamivir carboxylate was less than 5 % in rats, and a ethyl ester prodrug was designed, which is more hydrophobic and increased the bioavailability to 75 % [**58**].

2.7.3 Pharmacodynamics

2.7.3.1 *In vitro* antiviral activity

In enzyme assays, oseltamivir carboxylate inhibits NA of influenza A viruses with a K_i of 0.1-1.3 nM and influenza B viruses NA with K_i of 2.6 nM [**55**].
Concentrations of oseltamivir carboxylate required to inhibit NAs of other viruses and bacteria with NA are at least 10^5 times greater than that inhibit influenza NA [**57**].

2.7.3.2 Safety and toxicity in animals

Oseltamivir is well tolerated in mice, ferrets and rats. Mice tolerated doses up to 100 mg/kg/day and rats as much as 800 mg/kg/day. These doses, on weight basis, are 50 and 400 times, respectively, grater than the recommended therapeutic dose for adult patients [**57**].

2.7.4 Pharmacokinetics

2.7.4.1 Absorption, bioavailability, distribution, metabolism and elimination [55]

Oseltamivir is readily absorbed from the gastrointestinal tract after oral administration of oseltamivir phosphate and is extensively converted predominantly by hepatic esterases to oseltamivir carboxylate. At least 75 % of an oral dose reaches the systemic circulation as oseltamivir carboxylate.

The volume of distribution (V_d) of oseltamivir carboxylate by steady state is approximately 23 liters for humans, a volume which are equivalent to the extracellular body fluid.
The binding of oseltamivir carboxylate to human protein is low, only 3 %. The binding of oseltamivir to human plasma protein is 42 %, which is insufficient to cause significant displacement-based drug interactions [**42**]. Protein binding also slows the elimination of drugs that are removed from the serum by renal clearance. Oseltamivir carboxylate has a low protein binding and are therefore quickly removed from the circulation. Oseltamivir carboxylate must therefore be taken twice daily and possibly in larger doses than if the protein binding had been higher.

In vitro studies have shown that neither oseltamivir nor oseltamivir carboxylate is a substrate for, or inhibitor of, cytochrome P450 isoforms.

Absorbed oseltamivir is primarily (>90%) eliminated by conversation to oseltamivir carboxylate. Oseltamivir carboxylate is not further metabolized and is eliminated in the urine. Oseltamivir carboxylate is eliminated entirely (>99%). Renal clearance (18.8 l/h) exceeds glomerular filtration rate (7.5 l/h) indicating that tubular secretion occurs, in addition to glomerular filtration. Less than 20 % of an oral radiolabeled dose is eliminated in feces. The elimination is of first order kinetic and the half life of oseltamivir carboxylate is 6-10 hours.

2.8 AVIAN FLU

Aquatic birds provide the natural reservoir for influenza A viruses and are the source of all influenza viruses in other species, but in general, avian influenza is asymptomatic in wild birds. Only occasionally, highly pathogenic strains of influenza cause serious systemic infections in domestic poultry.

All avian influenza viruses are classified as type A, and are the key contributors to the emergence of human influenza pandemics. Different studies have defined two types of avian influenza A viruses based on their virulence: a highly virulent type that causes fowl plagues, and an avirulent type that causes only mild disease or asymptomatic infection. The history of highly virulent avian influenza viruses have clearly demonstrated that all of the pathogenic avian influenza A viruses are of the H5 or H7 subtype. There appears to be no association of NA subtypes with virulent viruses [**21**].

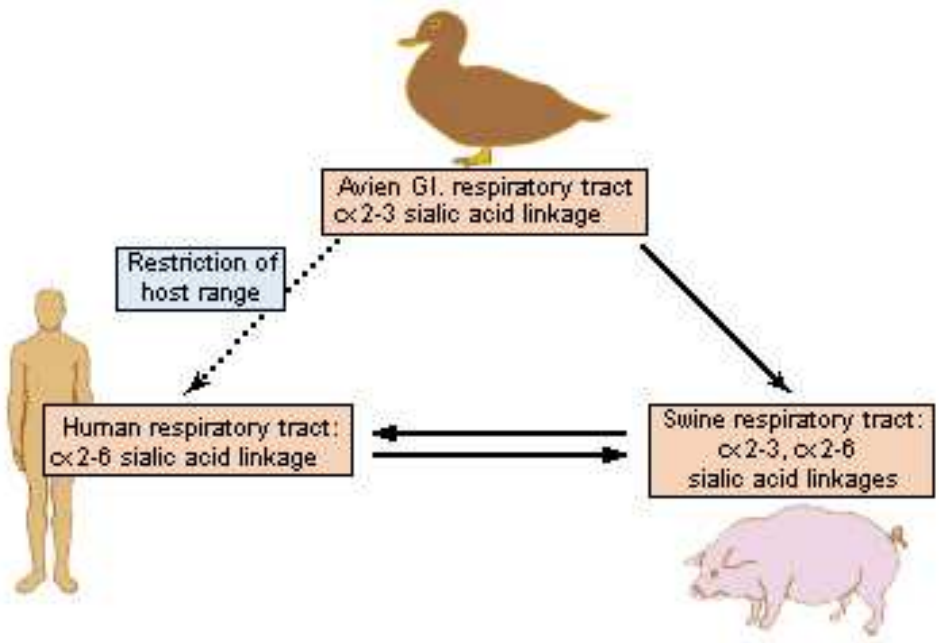


Figure 19 The avian, human and swine upper respiratory tract epitheliae preferentially express α 2-3 linkage, α 2-6 linkages and both linkages, respectively [59].

The avian influenza virus that appears most threatening now is the H5N1 strain, which recently is a growing concern in Asia. Before 1997, no evidence had indicated that the H5N1 viruses could infect humans and cause fatal disease. Now it has infected also cats, pigs and other mammals, often with fatal results. A human pandemic with H5N1 virus could potentially be catastrophic because most human populations have negligible antibody mediated immunity to the H5 surface protein and this viral subtype is highly virulent.

Influenza in avians is an infection of the gastrointestinal tract, with transmission predominantly through faeces. This fecally contaminated water-oral route is a major mechanism of virus transmission amoung aquatic birds and from migratory birds to domestic poultry over a wide area. The migratory birds are also important for the transmission of avian influenza between the Northern and Southern Hemisphere [**21**].

Influenza A viruses are carried asymptomatically in the gastrointestinal tract of wild birds, but may cause disease in domestic birds and in mammals such as humans and pigs. Similarly, human viruses do not replicate efficiently in waterfowl when introduced by natural routes. The tracheal epithelia of birds and humans mainly express influenza A receptors with an α 2-3 linkage and α 2-6 linkage of sialic acid, respectively, whereas pig tracheal respiratory epithelium expresses receptors with both α 2-3 and α 2-6 linkages [**60**].

The specificity of HA for sialic acid in α 2-3 or α 2-6 linkage to galactose is a key determinant in restricting the transfer of influenza virus directly from avian species to humans without mutations in HA sialic acid binding site occurring.

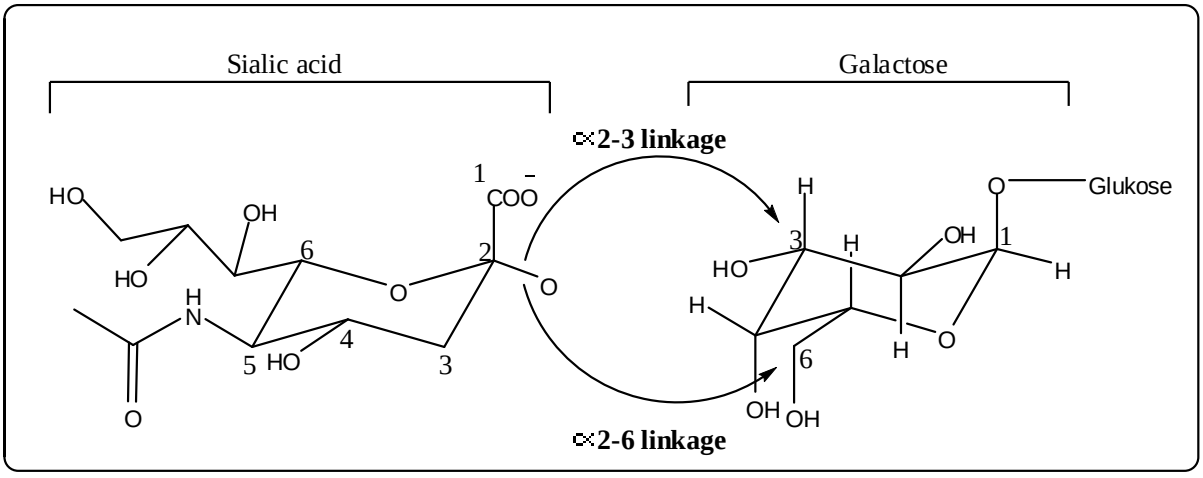


Figure 20 Sialic acid residues covalently attached to galactose residues via either α 2-3 or α 2-6 linkages

As of October 2006 the outbreak has resultet in 151 human deaths according to the World Health Organization, most of them in Asia and with history of exposure to sick and dead poultry in their neighbourhood [**61**]. Recent epidemiological evidence indicates that human-to-human transmission of avian H5N1 can occur. This is a growing concern because it suggests that only minor alternations in current avian H5N1 strains might result in efficient human-to-human transmission, a necessary condition for a human pandemic [**59**]. The pathogenicity of the virus has also made it difficult to produce an effective vaccine. The H5N1 influenza strain is so pathogenic that it could not be grown in embryonated eggs

because it killed the embryos before high levels of virus were produced [**7**]. In addition their virulence is a threat to the personnel in the vaccine production. The H5N1 strains are however sensitive to NA inhibitors and both national and international stockpiles of neuraminidase inhibitors are established for rapid response at the start of a pandemic.

2.9 COUMARIN

In this thesis coumarin was chosen as the pro moiety for the new potential prodrugs of oseltamivir carboxylate. The major advantage by using coumarin as pro moiety is that coumarin has been studied for more than 200 years [**62**]. The toxicity profile of coumarin is therefore well known and it is found to be relatively nontoxic in many clinical and laboratory studies [**63, 64**].

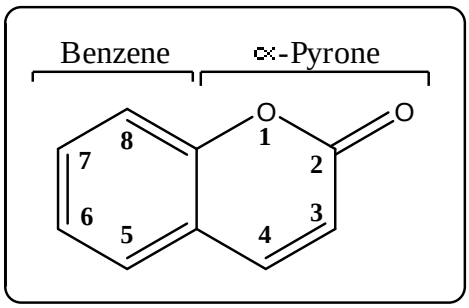


Figure 21 Coumarin

Coumarin is a naturally occurring secondary metabolite, being present in a widely variety of plants, microorganisms and in some animal species. Coumarin was first isolated from the seeds of the Tonka tree (*Dipteryx odorata*) [**62**]. The natural sources has never played a role in the isolation of coumarin, because the synthesis is simple and inexpensive [**65**]. Coumarin is used widely as a fixative and enhancing agent in perfumes and is added to toilet soap and detergents, toothpaste, tobacco products and some alcoholic beverages. Large quantities are also used in rubber and plastic materials and in paints and sprays to neutralize unpleasant odours.

Figure 22 Picture of the Tonka tree and dried tonka beans

The metabolism of coumarin displays large interspecies differences, with the rat demonstrating enhanced susceptibility to hepatic injury due to the 3,4-epoxidation pathway. The major metabolic pathway in humans is to 7-hydroxycoumarin via cytochrome P450 2A6. The 7-hydroxycoumarin is excreted in conjugated form, almost completely as glucuronide in the urine [**65**]. Since very few drugs are substrate for the CYP 2A6 isoenzyme, drug interactions involving this enzyme are therefore not likely to occur.

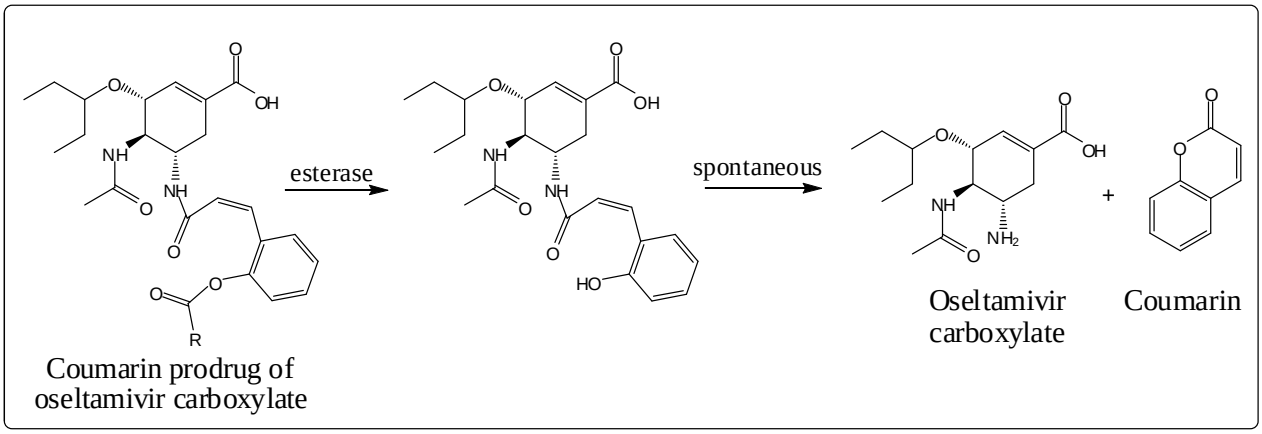


Figure 23 Illustration of the coumarin prodrug concept

2.10 PRODRUG

Definition:

"Prodrugs are chemicals with no pharmacological activity requiring biotransformation within the body in order to release the therapeutically active metabolite [**66**]*."*

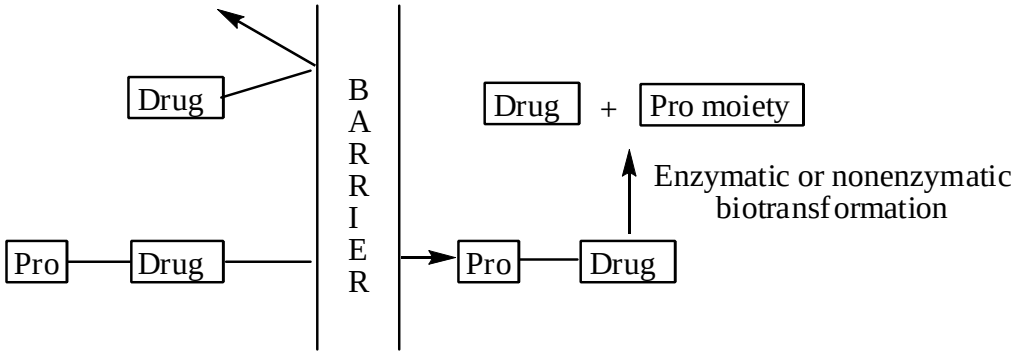


Figure 24 Schematic illustration of the prodrug concept [67]

The prodrug concept was introduced in 1958 by Adrien Albert [**68**]. And as defined, prodrugs are therapeutic agents that are inactive *per se* but are predictably transformed into active metabolites. The prodrug strategy is important for improvement of the physiochemical and biological properties of an parent molecule. A necessary requirement of this approach is the capability of the drug to revert to the parent drug in the body by enzymatic or chemical action, quantitatively and at a desirable rate.

There are many barriers a drug has to overcome, before it can be released at the desired time and site in a biological system [**69**]. Some of them are mention below:

Pharmaceutical	**Pharmacokinetic**	**Pharmacodynamic**
- irritation or pain	- incomplete absorption	- toxicity
- chemical instability	(high/low lipophilicity)	- adverse effects
- low/high lipophilicity	- short duration of action	
- offensive taste or odor	- low or variable bioavailability	
- poor aqueous solubility	- marked presystemic metabolism	
	- unfavorable distribution in the body	
	(lack of site specificity)	

Prodrugs can be obtained by direct attachment of a functional group to the pro moiety molecule. In the rational design of prodrugs, three basic steps have to be considered. That is the identification of the parent drug delivery problem, the physiochemical properties required for maximum efficacy or delivery and the choice of prodrug moiety. The chosen pro moiety should provide a prodrug which exhibit the proper physiochemical characteristics and which can be cleaved in the desirable biological compartment [**66**]. The conversation of prodrug to parent drug is mediated either by enzymes (esterases and lipases) or by pH.

The most common type of prodrugs are esters derivative from hydroxyl or carboxyl groups present in the parent drug molecules, but there are many other bioreversible derivatives for many other functional groups. The reason for the popularity of ester as prodrug is the fact that the organism is rich in enzymes capable of hydrolyzing ester bonds, they are ubiquitous distributed and several types are found in the blood, liver, and other organs and tissues. By appropriate esterification it is possible to obtain derivatives with almost any desirable aqueous solubility or lipophilicity [**66**]. While there has been an abundance of work done on ester prodrug, much less work has been completed on prodrugs of amids [**70**]. This is due to the relatively high stability of amids *in vivo*, which makes the regeneration of the original drug more difficult. The prodrug approach has also been successfully applied on a variety of other drugs to design derivatives, but these are not further mention here.

Application of the prodrug approach:

- improved formulation (e.g. modify aqueous solubility, mask bitter taste)
- improved chemical stability
- improved patient acceptance and compliance
- improved bioavailability
- improved organ selectivity
- site specific bioactivation
- decreased side effects
- marketing considerations
- prolonged duration of action (slow release of the parent drug from a prodrug, decreased first-pass metabolism of the drug)

2.10.1 Pharmacokinetic aspects of prodrug design

The pharmacokinetic of a prodrug is dependent on several factors, some of them are percentage of protein binding, rate of the esterase breakdown and clearance from the body.

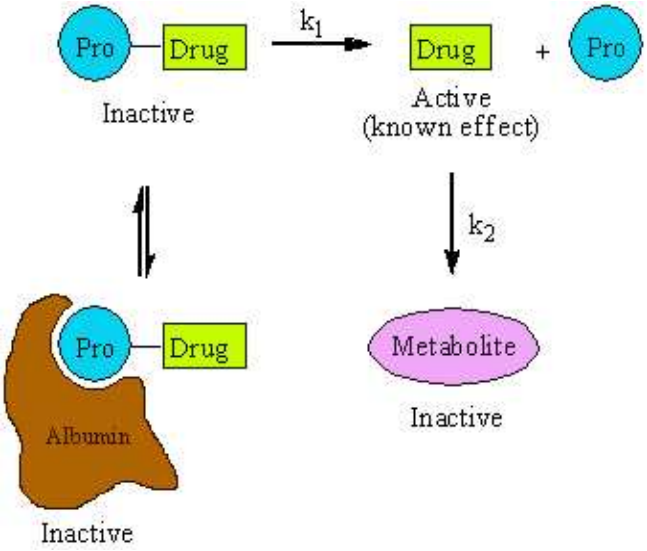


Figure 25 Pharmacokinetic of a protein bond prodrug
The amount of free prodrug available for hydrolytical esterase breakdown is in equilibrium with the amount of prodrug bound to albumin. K_1 is the rate of esterase breakdown, while k_2 is the clearance rate of the active drug.

Following is a hypothetically example of the pharmacokinetic of a prodrug:

A drug has a therapeutically window between 2-10 μg/ml (plasma concentration), the maximum bolus dose gives a plasma concentration of 10 μg/ml. The prodrug is nontoxic and can be administrated in amounts giving a plasma concentration of up to 0.6 mmol/l. At this concentration half of the binding capacity of albumin is saturated and displacement of other compounds is not likely to occur [**51**]. The kinetics is of 1^{st} order, with the value k_1, and the belonging k_2 is 0.5 $hours^{-1}$. As indicated in Figure 25 k_1 is dependent on the protein binding and the rate of esterase breakdown. The drugs volume of distribution of highly protein bound drugs is low.

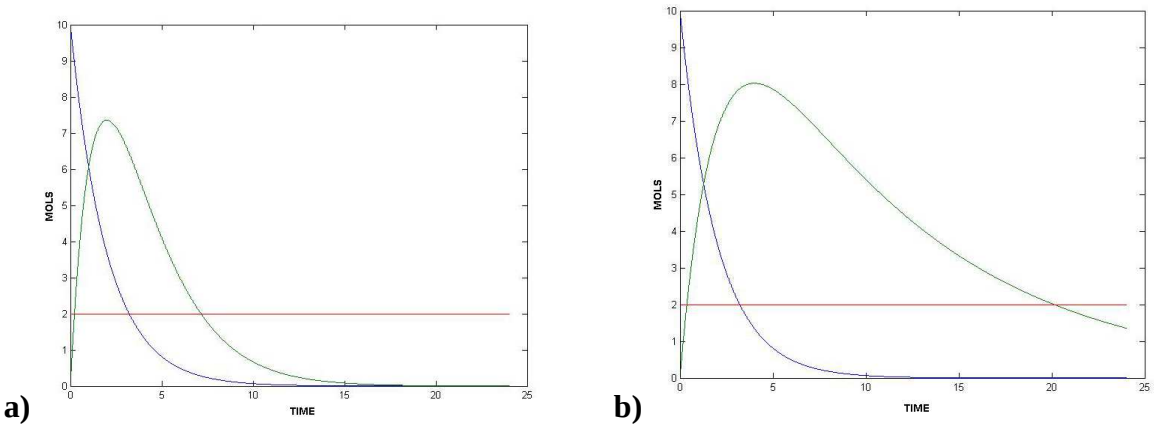


Figure 26 Hypothetically time courses following intravenous administration of drug and prodrug
Blue = drug followed an i.v. dose of drug, green = drug followed an i.v. dose of prodrug and red = lowest part of the therapeutic window

The Figure 26a) above the value of k_1= 0.5. It display the injection of twice the molar amount of prodrug compared to injected parent drug, the concentration of drug from prodrug never rises above the upper part of the therapeutic window. The concentration of drug from prodrug also lies twice as long inside time therapeutic window as the parent drug. In case of Figure 26b) the value of k_1=0.1. Six times the molar amount of prodrug is injected compares to the parent drug. The concentration of drug from prodrug stays also here inside the therapeutic window and has approximately 5 times longer duration of effect without exceeding the upper part of the therapeutic window.
In summary, the hypothetically example in Figure 26 shows that a prodrug can have major advantage compared to the parent drug. It can be administrated in a larger dose which gives a longer duration of effect without exceeding the therapeutic window. The patients compliance will therefore increase, as the frequency of dose administration decreases. The prodrug might be administrated only once dose a day, once a week or one dose might be sufficient for the hole treatment, which often is the case for Azitromax® which, due to active metabolites and the drug itself, stays in the body for several days.

Oseltamivir is the ethyl ester of its active metabolite and is hydrolyzed due to extensive first pass metabolism, which is relatively normal for simple ester prodrug. Certain drug, for example pivampicillin, is design as prodrugs to increase the oral bioavailability and the fast hydrolysis to its active metabolite is advantageous. This is similar to oseltamivir, the ethyl ester was designed to increase the oral bioavailability, not its duration of effect. In this thesis, amid prodrug is designed to increase the binding to human serum protein. These prodrugs are

among the few which have a free carboxylic group and the strategy is to investigate if these prodrugs can avoid the first pass metabolism and remain in the circulation for a longer time then oseltamivir. To confirm that this might be the case for these new prodrugs, many further studies have to be preformed.

2.10.2 Pro- prodrugs

Figure 27 Schematic illustration of the pro-prodrug concept

A pro-prodrug is a derivative which must undergo two independent reactions in order to regenerate the parent drug. The prodrug intermediate possesses a high chemical reactivity, allowing it to rapidly convert to the parent drug. This chemical reaction cannot occur before the biological conversation of the pro-prodrug (a chemically stable molecule) to its prodrug form. Since simple amids are generally are too stable *in vivo* to be useful prodrugs forms for amines, many investigators have attempted to prepare amides which utilize an intramolecular reaction (e.g. lactonization) to increase the lability of the amide bond, thus facilitating release of the amine [**66, 71**]. A strategy to overcome this problem is the pro-prodrug concept.

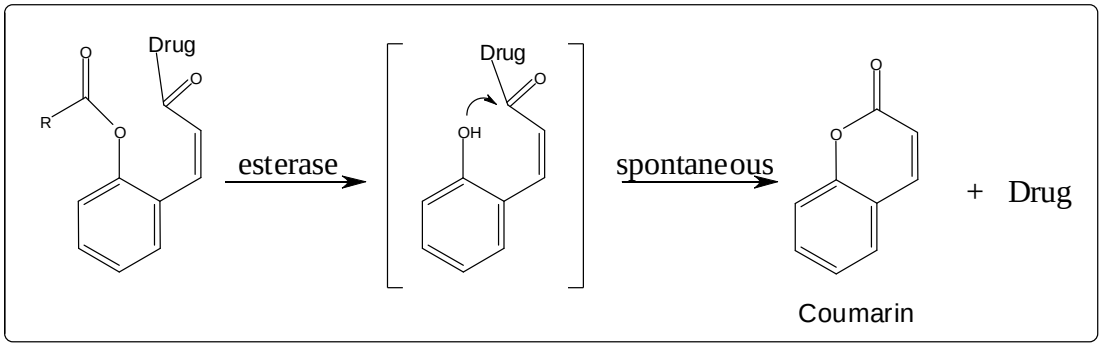


Figure 28 The concept of a coumarin based esterase sensitive prodrug system

This prodrug concept takes advantage of intramolecular cyclization reactions.

The design of this prodrug system takes advantage of the facile lactonization of cinnamic acid and its derivatives. But the major advantage of this system is that the toxicity profile of coumarin is well-known and coumarin has been found to be relatively non-toxic in many

clinical and laboratory studies [**63**]. It is a pro-prodrug concept were the pro-prodrug form is susceptible to esterase metabolism, and the prodrug can there by undergo spontaneous cyclization, regenerating the drug and the coumarin pro moiety.

2.11 PROTEIN BINDING

Many drugs bind to proteins in plasma. Albumin is the most important drug binding protein although α_1-acid glycoprotein, β-globulin, α_1-globulins, α_2-globulins and lipoproteins also binds and carry a wide variety of drugs.
The drug binding to plasma and tissue proteins are fundamental factors in determining the overall pharmacological activity of a drug. And the drug binding depends on three factors; the concentration of free drug, its affinity for the binding sites and the concentration of protein. It is the unbound drug that is pharmacologically active and can undergo metabolization. Therefore the extent of binding of a drug to plasma protein controls both its effect and the duration of the effect. Competition between drugs that binds to the same site in albumin is a frequent cause of drug interaction and can cause serious toxicity owning to the increased concentration of the drugs free form. This is the case for drugs that has relatively narrow range of therapeutic concentration and are more than 90% bound to albumin [**72**]. And there are few drugs that affect binding of other drugs because most of them occupy, at therapeutic concentration, only a tiny fraction of available binging sites.

2.11.1 Albumin

The name albumin origins from the Latin word *albus*, which means white. More general it evolved from the term *albumen*, which is an early German word for protein. The album*en* is still used in for the white of an egg, for the secretion of the snail and for urinary proteins as a group. The album*in* is specifically used for protein from blood plasma or to a protein with similar properties [**72**].

Human serum albumin (HSA) has a molecule mass of approximately 66.5 kDa and is the most abundant protein in blood plasma. Albumin accounts for approximately 60% of all plasma proteins and the usually concentration is about 0.6 mmol/l in the bloodstream.

Albumins ability to bind smaller molecules of many types is well known, and the most studied ligand which binds to albumin is bilirubin. The albumin molecule has two primary binding sites, Sudlows site I and II, but several other binding sites for drugs has also been identified on the protein. These other binding sites have a lower binding affinity and selectivity.

Figure 29 Three domain structure of HSA [73]

Sudlows site I binds mainly hydrophobic, bulky, heterocyclic molecules with a centrally located negative charge (e.g. warfarin, phenylbutazone).

Sudlows site II binds mainly aromatic carboxylic acids with a negative charge at one end of the molecule distal from the remaining hydrophobic structure (e.g. diazepam, ibuprofen).

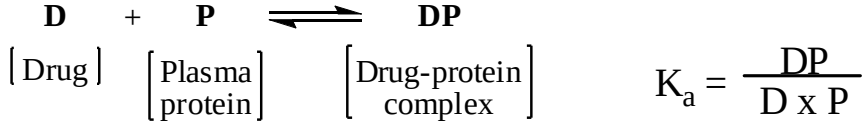
One of albumins main functions is transporting endogenous and exogenous (e.g. drugs) compounds and it act like a buffer on the free drugs concentration **[73]**. The protein binding of endogenous compounds (such as hormones) exhibits higher affinity and specificity than those of exogenous compounds. Binding of drugs to albumin alters the pattern and volume of distribution, can lower the rate of clearance and increases the plasma half life of the drug. These characteristics can either be a benefit or a drawback for the drugs efficacy. A drugs binding mechanism to plasma protein is therefore essential to understand the pharmacokinetic, pharmacodynamic and toxicological profile of any drug.

Human albumin is also used therapeutically in hypovolemic states like shock (due to recent blood loss), burns or under surgery [**72**]. This human albumin product was developed under

World War II in the treatment of shock among soldiers on the battlefield, but after the peace was restored, this treatment strategy was quickly adopted by surgeons in civilian hospitals.

2.11.2 Binding equilibrium

The binding reaction of a drug to plasma protein can be regarded as the association of drug molecules with the plasma protein.



(Equation 1)

The association constant (K_a) will be large for drugs with high protein binding and small for drugs with low protein binding. The two primary binding sites provide the albumin molecule with a binding capacity of about 1.2 mmol/l. The normal plasma concentration of most drugs is much less then 1.2 mmol/l, and the binding sites are therefore far from saturated. This reveals that most binding of drugs to albumin are independent of drug concentration [**51**]. Drugs that work at plasma concentration where the protein is approaching saturation is very sensitive to further increase in the concentration. Because this will leads to an enormously grater amount of free drug in the plasma and may cause side effects and possibly intoxication.

2.11.3 Protein binding prodrugs

Designing prodrugs that has higher protein binding than its parent drug is a potential to cause drug interactions. This is because a drug with high protein binding can cause displacement of another drug which also binds highly to proteins and cause side effects or even lack of effect due to a faster clearance. However, there are very few examples of clinically important drug-drug interactions which are exclusively due to displacement of drugs from the plasma protein binding sites [**6**]. Other processes like absorption, hepatic metabolism and renal clearance are of far greater importance to the drug to drug interactions then plasma binding displacement [**74**]. Only when the concentration of one of the drug approaches the molar concentration of the binding sites substantial displacement will occur. And because most drugs are relatively potent, the binding sites are far from saturated. However displacement interactions may be of clinical importance if the displaced drug has a small apparent volume of distribution, narrow therapeutic index and rapid onset of action.

High protein binding of a prodrug favors site directed drug delivery due to increased permeability in inflammatory tissue. Invading pathogens, like influenza viruses, causes activation of the innate immune response immediately after the infection. This activates a cascade of events which include production of pro-inflammatory cytokines. These cytokines acts on vascular endothelial cells of the postcapillary venules and causes an increased permeability in the area of inflammation [**51**]. This enhanced vascular permeability in inflammatory tissues causes extravasation of macromolecules, including plasma proteins and liposomes. Prodrugs with high protein binding would therefore get a specific target effect to the inflammatory site.
This enhanced permeability is also seen in tumor tissue, in addition to enhanced retention and the effect has been described enhanced permeability and retention effect (EPR). However, enhanced retention is not seen in inflammatory tissue because the clearance of macromolecules and lipids from the interstitial space of inflammatory tissue function as normal via the lymphatic system. In tumor tissue this clearance via the lymphatic system is impaired and enhanced retention is therefore seen [**75, 76**].

The plasma protein binding is relevant in several different pharmacokinetic models. The volume of distribution, V_d, indicates in what compartment the drug will be confined to, tissue or plasma. A drug with high protein binding will have a low V_d, because the drug would be confined to the plasma compartment to larger extent. This may minimize adverse affects and give a targeting of the drug to the inflamed tissue.
All clearance models have also incorporated the protein binding term. The clearance of a protein with high protein binding will be slower than if the drug was found as free, unbound drug in the plasma. And again the half life of a drug is dependent on the rate of clearance. So higher plasma protein binding leads to lower V_d, lower clearance and longer half life.

3 AIM OF THE STUDY

3.1 MAIN AIM

The main aim in this thesis is to study different synthesis strategies around some novel prodrugs of the neuraminidase inhibitor oseltamivir carboxylate (GS4071).

3.2 SUB AIM

3.2.1 Hydrolysis of oseltamivir phosphate

The hydrolysis of oseltamivir phosphate to oseltamivir carboxylate should be studied and a condition giving a good yield of oseltamivir carboxylate and a minimum of byproducts should be found.

3.2.2 X-ray crystal structure

Crystals of oseltamivir phosphate and oseltamivir carboxylate should be grown and used for studies to determine the x-ray crystal structure and to verify the final hydrolysis product of oseltamivir phosphate.

3.2.3 Selection of pro moieties

A nontoxic pro moiety with potential use in the design of amid prodrug of oseltamivir carboxylate should be chosen and evaluated. A series of different ester of this pro moiety should be synthesized and used as potential prodrug candidates. The synthesized amid prodrugs should contain a free carboxylic acid which might increase the prodrugs protein binding.

3.2.4 Selection of synthesis strategy

Different strategies for synthesizing prodrugs of the chosen promoieties should be studied.

3.2.5 Further studies of the new amide prodrug

After synthesizing the new prodrugs of oseltamivir carboxylate, potential further studies should be outlined. The binding to human serum albumin, stability towards different esterases (serum- and hepatic esterases), penetration through cells and different animal model assays would be studied.

4 RESULTS AND DISCUSSION

4.1 THE PRODRUG STRATEGY

Oseltamivir is the ethyl ester prodrug of oseltamivir carboxylate. Oseltamivir has a high bioavailability (75%) in capsule and suspension formulations. The protein binding of oseltamivir is 42%, but is of minor importance due to the fast breakdown of hepatic esterases. The active metabolite oseltamivir carboxylate has a very low protein binding of less than 3%. By synthesizing some new prodrugs of oseltamivir carboxylate which has a higher protein binding, this new prodrug might have many advantages in proportion to oseltamivir.

A higher protein binding might lead to:

- a prolonged duration of action, due to sustained release effect of albumin bound drug
  - improvements in the patients acceptance and compliance, decreased administration of doses
- improved organ selectivity, because of the enhanced permeability in inflammatory tissue
- marketing considerations

In this thesis, different coumarin prodrugs of oseltamivir carboxylate were attempted synthesized. From the theory, these prodrugs would have a higher protein binding due to more hydrophobic characteristics and a free carboxylic group. Coumarin is also a well studied compound which is nontoxic. These coumarin prodrugs take advantage of an intramolecular lactonization, which rapidly convert the prodrug back to oseltamivir carboxylate and coumarin.

Table 2 Expected logP values for some of the potential coumarin prodrugs[2]

	CLogP
Oseltamivir	2.13
Oseltamivir carboxylate (deprotonated)	-1.43
R = $-CH_3$	0.43
R = $-CH_2CH_3$	0.96
R = $-C_6H_6$	2.56

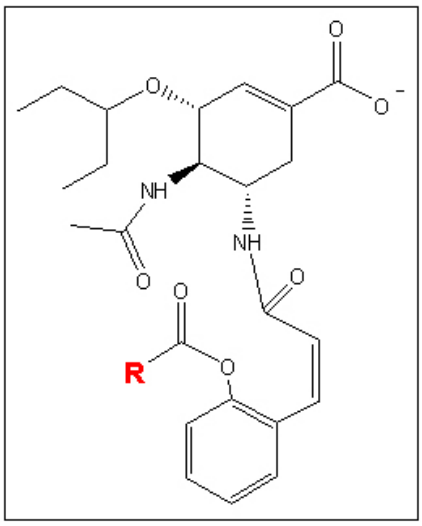
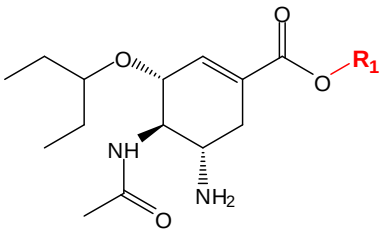


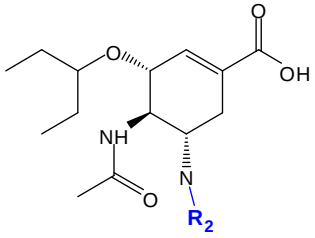
Table 2 indicates the expected logP values for some of the potential coumarin prodrugs. According to Lipinski *et al.* compounds with logP values below 5 can be expected to have a good absorption and permeation. In addition the compounds should not have more then five hydrogen bond donors, molecular weight under 500 and not more then 10 hydrogen bond acceptors [**77**]. These prodrugs have a free carboxylic group and their binding to albumin have to be measured in albumin binding assays. A compound with low CLogP and a free carboxylic acid might just as well have a higher protein binding then one with higher CLogP which lack a negative charge *in vivo*. This would have been investigated if the prodrugs had been synthesized.

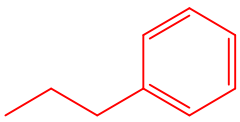
Structure search in the database SciFinder® Scholar™ of different analogs of oseltamivir carboxylate revealed that coumarin prodrugs of oseltamivir carboxylate has never been synthesized before. Only 14 compounds were found, 7 amid prodrugs and 7 ester prodrugs (Table 2 below). Most of these compounds, except compound number II (oseltamivir) and IX, exist only in patents. Because these patents were written in Japanese, further studies around whether these compounds have been synthesized or not became very difficult.

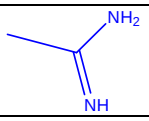
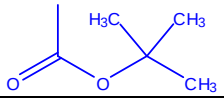
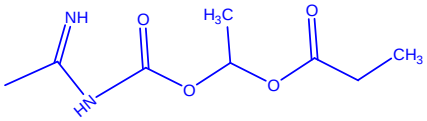
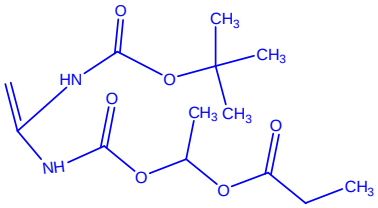
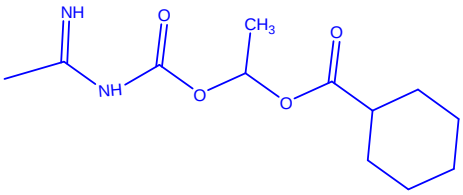
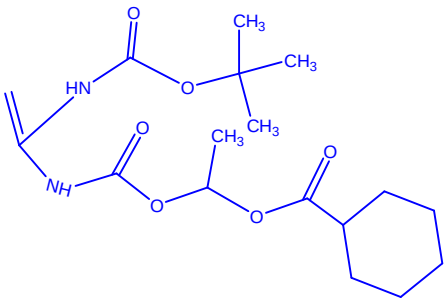
[2] CLogP values are calculated from ChemDraw Ultra 10.0

Table 3 Known substances of oseltamivir carboxylate[3]



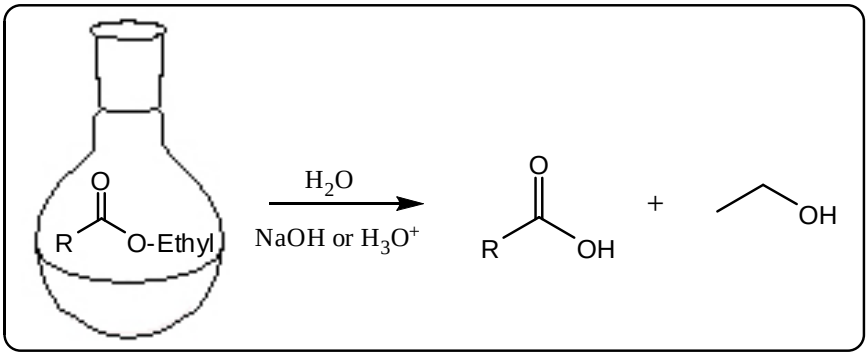


Registry number	R_1	
208720-71-2	—CH_3	I
196618-13-0 Oseltamivir GS 4104	—CH_2CH_3	II
736122-77-3	—$CH_2CH_2CH_3$	III
732972-20-2	—$CH_2CH_2CH_2CH_3$	IV
371193-37-2	—$(CH_2)_{13}CH_3$	V
371193-39-4	—$(CH_2)_{17}CH_3$	VI
790193-12-3		VII

Registry number	R_2	
221386-92-1	—CH_3	VIII
208589-55-3		IX
764639-63-6		X
475482-78-1		XI
475482-81-6		XII
475482-84-9		XIII
475482-86-1		XIV

[3] Based on structure search in SciFinder® Scholar™, September 2006

4.2 SYNTHESIS OF OSELTAMIVIR CARBOXYLATE



The ethyl ester of oseltamivir can be cleaved to the carboxylic acid oseltamivir carboxylate in both aqueous base and aqueous acid. Hydrolysis of oseltamivir gives the active metabolite oseltamivir carboxylate and ethanol.

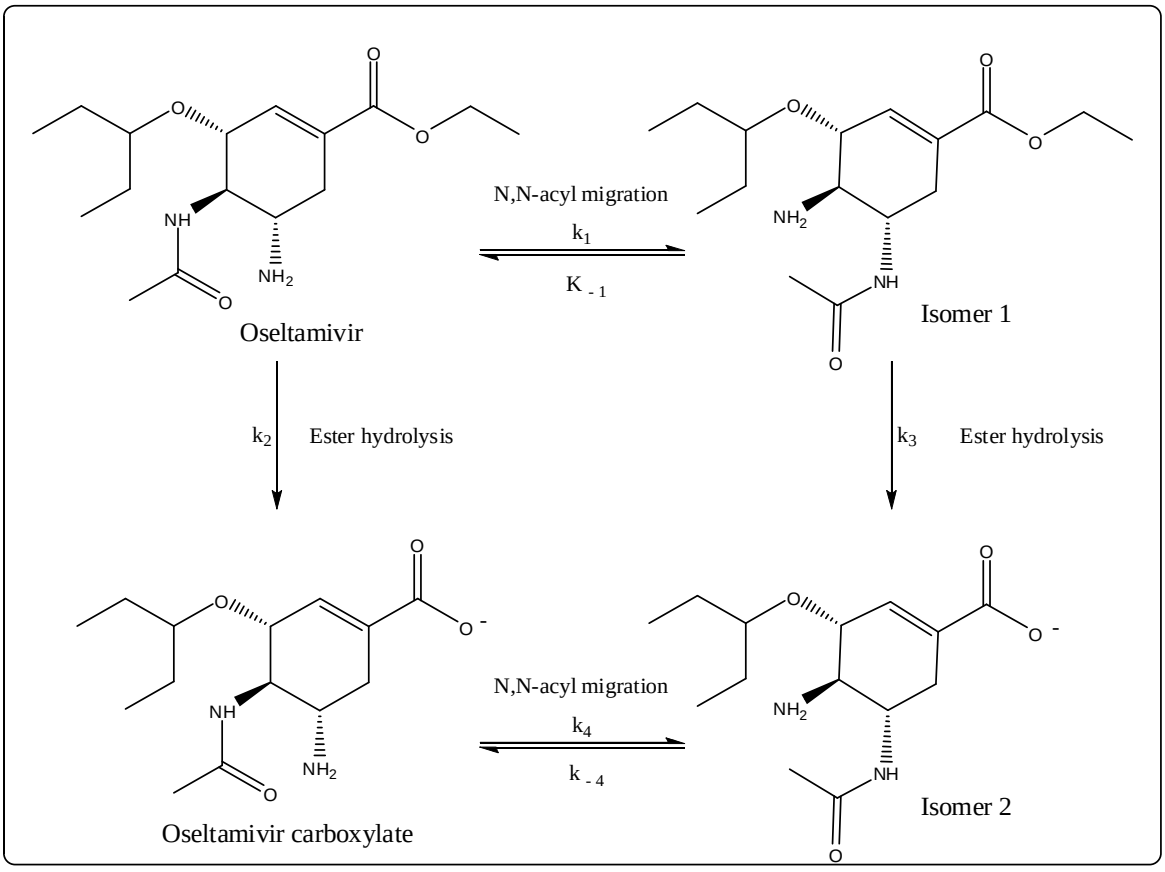
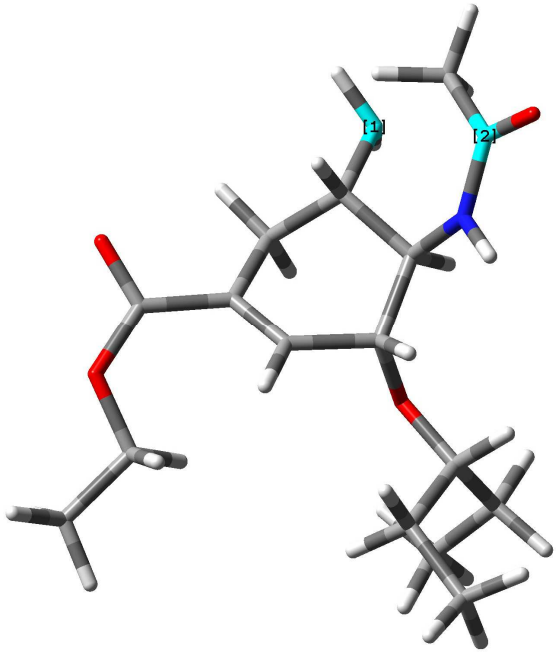


Figure 30 Degradation pathways for oseltamivir [58]

The pathways for the degradation of oseltamivir in aqueous acid solution have been described by Oliyai *et al.* (Figure 30). Two degradation pathways were identified for oseltamivir, the

N,N-migration of the acyl group (yielding isomer 1) and the ethyl ester hydrolysis (yielding oseltamivir carboxylate) Because oseltamivir is prone to acyl migration under acid condition [**78**], the formation the degradation products isomer 1 and 2 develop in addition to oseltamivir carboxylate. Unlike the degradation study in Oliayi *et al.*, this thesis hydrolysis of oseltamivir was preformed at room temperature and not at 70 ºC. The 3D structure of oseltamivir was studied in the program Gauss View 3.07, after optimizing the structure with MM2 force field in Chem3D 10.0. The amino group (atom number 1) and the acyl group (atom number 2) were rotated into their closest direction (Figure 31). Even though they are rotated as close as possible they did not get any closer than ≈ 2.95 Å, this is because they are in *trans* positions.



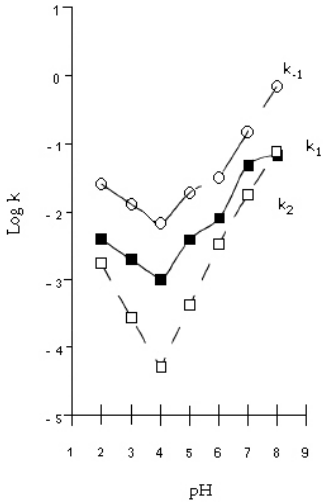
This explains why hydrolysis at room temperature might not have the same problem with acyl migration as hydrolysis at 70 ºC, because less kinetic energy is available at room temperature to overcome the activation energy for acyl migration.

Results from HPLC and NMR analysis confirm that acyl migration is not any problem in the room temperature hydrolysis.

Figure 31 Oseltamivir

The amino group and the acyl group of oseltamivir are rotated into their closest direction. The numbers shows the amino nitrogen (1) and the carbonyl carbon (2).

Figure 32 shows the pH rate profiles described in Oliyai *et al.* This figure indicates that pH 4 would give the lowest value for k_2, which increases for more acidic and more basic values. The degradation reactions of oseltamivir would go more extensively towards the side reactions at pH 4 (Figure 30 above).



In this thesis, the hydrolysis was preformed at pH < 2. A hypothetic extrapolation of the rate constants down to values below pH 2, at ambient temperature, the k_2 might increase to a level fare above k_1 and k_{-1}.

Acid condition for the hydrolysis of oseltamivir was therefore chosen for the first attempt.

Figure 32 Plots of the pH rate profiles for the isomerization (k_1 and k_{-1}) and ester hydrolysis (k_2) of oseltamivir at 70 °C[58].

4.2.1 Selection of acid concentration

Studies of aqueous acid hydrolysis of oseltamivir phosphate were made with three different concentrations of HCl, 15%, 25% and 32%. These studies revealed that 25% HCl gave the highest relative absorbans of oseltamivir carboxylate and lowest absorbans of impurities. Use of stronger acid than 25 % only increased the absorbans for impurity, while hydrolysis with weaker acids gave a decreased absorbans of oseltamivir carboxylate (Figure 33).

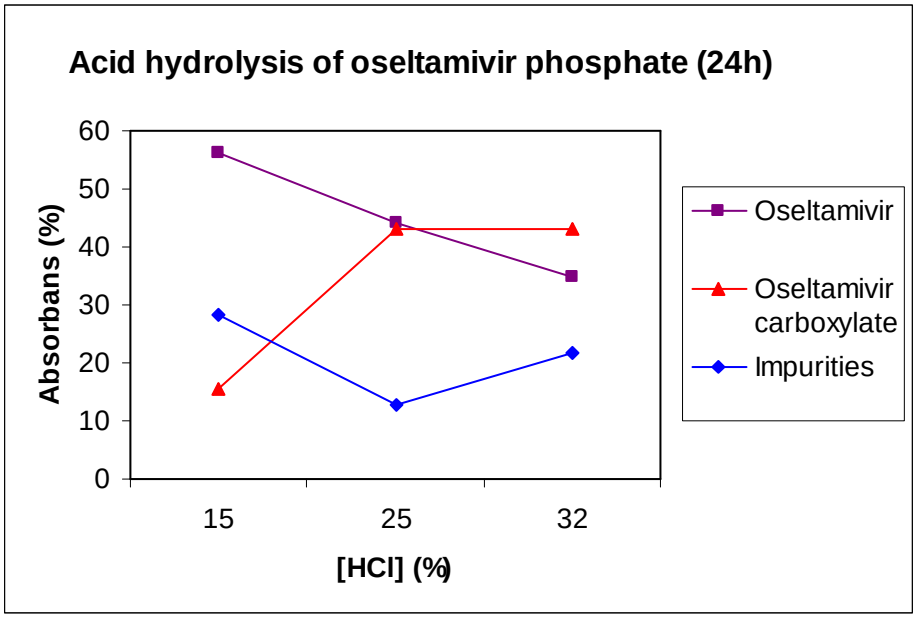


Figure 33 Diagram showing the absorbans of oseltamivir carboxylate after 24h of aqueous acid hydrolysis[4].

4.2.2 Selection of reaction time for the hydrolysis

After chosen the acid concentration for the hydrolysis, the reaction time was investigated. The hydrolysis of oseltamivir phosphate was studied over 5 days in 25 % HCl (Figure 34 below). These studies indicated that the HPLC peak of oseltamivir carboxylate increased over time and after 5 days over 50% of the oseltamivir phosphate was hydrolyzed. The reaction time was therefore chosen to 5 days, which gave the highest relative absorbans of oseltamivir carboxylate.

[4] Diagram based on results from HPLC analysis.
Absorbans measured on HPLC (G1) at 230 nm.

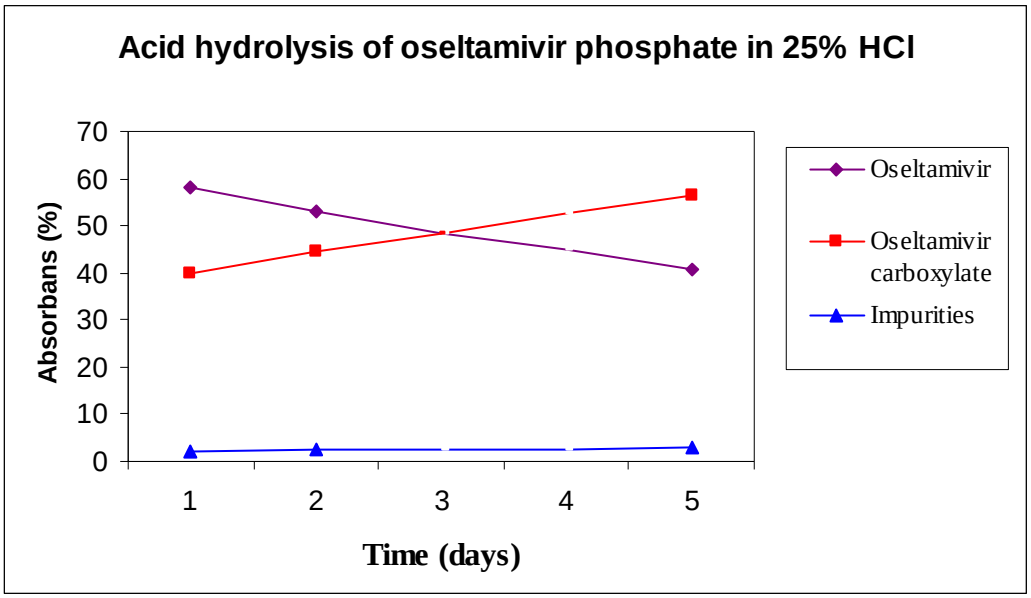


Figure 34 Acid hydrolysis of oseltamivir phosphate over time[5]

After several attempts to purify the oseltamivir carboxylate that had been hydrolyzed over 5 days, it revealed that even though this reaction time might have given the highest relative absorbans of oseltamivir carboxylate in the HPLC assay, the product was very difficult to isolate. Oseltamivir has probably been broken down to undetectable products in the HPLC assay (view point 4.2.4 below). Purification of oseltamivir carboxylate after just 1 day of hydrolysis solved this problem, so further hydrolysis were neutralized and purified after 1 day reaction time (further described in section 4.2.3 below). The yield of purified product is well comparable with the relative absorbance in the HPLC assay after 1 day of hydrolysis.

4.2.3 Purification of oseltamivir carboxylate

The purification of oseltamivir carboxylate was preformed by preparative HPLC after 5 days hydrolysis. The analytical HPLC showed that the oseltamivir carboxylate were sensitive to eluents with HCOOH (aq), so further attempts to separate the peaks were done with just H_2O in the eluent. Formic acid pH 3 (HCOOH (aq)) in the eluent protonates the amine group and the molecule is positive charged. The molecule had therefore no retention time on in the HPLC assay and was washed out together with the eluent, a neutral eluent was therefore used. Even though the analytical HPLC showed symmetrical and well separated peaks with ACN/H_2O (1:9), the purification process on the preparative HPLC gave no yield.

[5] Diagram based on results from HPLC analysis of several studies.
Absorbans measured on HPLC (G1) at 230 nm.

Attempted eluents:

- ACN/H_2O (1:9)
- ACN/Ammonium acetate, 0.05 M (aq) (1:9)
- ACN/Hydroxylamine, 3 % (aq) (1:9)

Neither of these eluents was successful and the yield after purification was approximately equally to 0. The eluent with hydroxylamine gave fractions with product, but further analysis on HPLC showed a peak with shorter retention time than oseltamivir carboxylate. This indicates that something might have happened under the purification process and that oseltamivir carboxylate might not be stable in solutions with hydroxylamine.
Purification of oseltamivir carboxylate with ammonium acetate in the preparative HPLC eluent gave a small amount of product, but less than acceptable. Even though this purification procedure has been described by Oliyai *et al*, it did not work in this case. And if it had worked, the collected eluent from this purification had to be freeze dried twice to remove the excess amount of the ammonium acetate salt [**58**].

After several attempts to purify oseltamivir carboxylate that had hydrolyzed for 5 days, new attempts were made with reactions products that had just been hydrolyzed for 1 day.
These reaction products revealed different characteristics and the yield after preparative HPLC was much better. Overall, the yield from purification with ACN:H_2O (1:9) was approximately 47 %. This yield also correlates to the relative absorbans measured in the HPLC analysis of the reaction mixture.

4.2.4 Degradation products of oseltamivir phosphate

The hydrolysis of oseltamivir phosphate to oseltamivir carboxylate in 25 % HCl can result in degradation to other unwanted products.

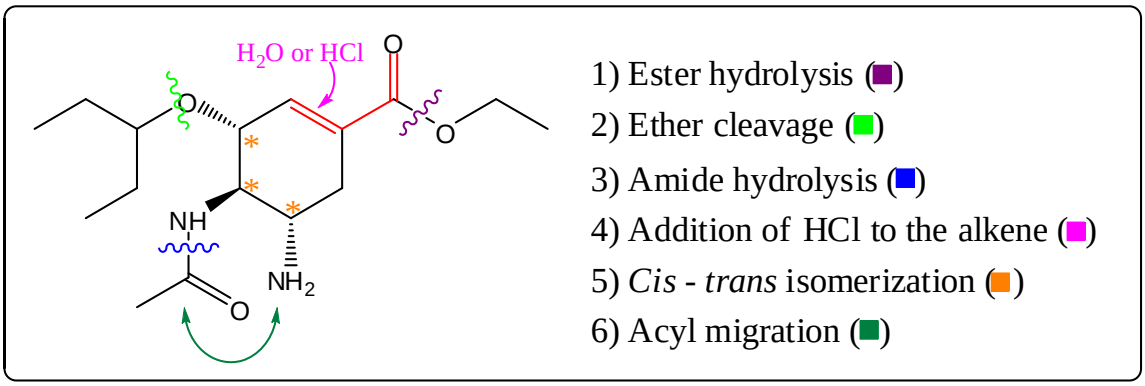


Figure 35 Possible degradation reactions that can occur of oseltamivir phosphate under acid condition (red = chromophore group)

As shown in the figure above, there are several different reactions that can occur under acid condition:

1) Ester hydrolysis

   This is the wanted reaction, which yield the oseltamivir carboxylate.

2) Ether cleavage

   Under very acid conditions, the ether group can be cleaved to give two alcohol molecules.

3) Amide hydrolysis

   Hydrolysis of the amide gives an amine and a carboxylic acid.

4) Addition of H_2O or HCl to the alkene

5) *Cis – trans* isomerization

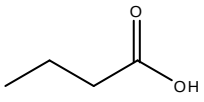
   Isomerization of the stereo centers can occur under acid condition.

6) Acyl migration

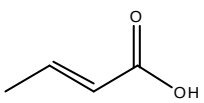
   Acyl migration has been described in the literature for acid condition at 70 ºC.

7) Polymerization

In a HPLC assay with neutral eluent, for example ACN:H_2O (1:9), all these degradation pathways can be detected, except reaction number 4. All the compounds will have different retention time and the chromophore group is intact. In reaction 4, the conjugated chromophore group is lost. Absorbtion from the carbonyl double bond is best observable when it is conjugated with another double bond. Saturated carboxylic acids have weak absorbans around 200-215 nm, with a molar absorptivity (ε) of about 30-100, therefore the absorption often goes unnoticed. Conjugated acids show much stronger absorptions, one C=C double bound conjugated with the carboxyl group results in a spectrum with λ_{max} still around 200 nm, but with molar absorptivity of about 10,000 [**79**].



λ_{max} = 200 - 215 nm ε = 30-100



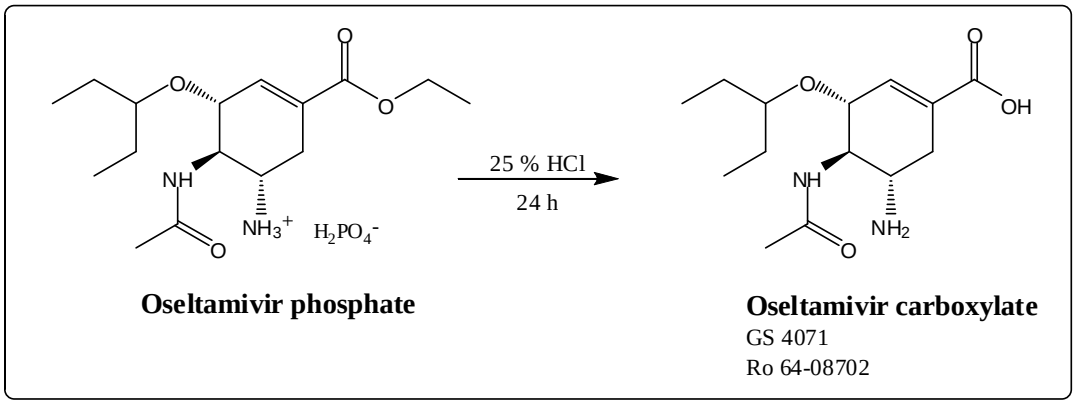
λ_{max} = 200 nm ε = 10,000

Figure 36 Molar absorptivity of different carboxylic acid

All these 7 reactions can be verified by NMR. Reaction 5 which gave isomerization can be detected by analyzing the couplings constant in the ^{1}H NMR spectra.

In the analytical HPLC assay used in the experimental section, I1, all compounds with longer retention time then 10 minutes were not detected, neither were compounds without the chromophore group.

4.2.5 Summary of the chosen strategy for hydrolysis of oseltamivir phosphate



Oseltamivir phosphate

Oseltamivir carboxylate
GS 4071
Ro 64-08702

The hydrolysis of oseltamivir was preformed with 25% HCl for 24 hours.
ACN:H_2O (1:9) was chosen as eluent for the purification on preparative HPLC. The fractions with retention time of 4.6 minutes (I1) was collected and further analyzed by NMR and MS. The analyze results from NMR and MS revealed that the isolated product was pure oseltamivir carboxylate.

To verify that the stereo centra are preserved, the couplings constant in the ^{1}H NMR spectra was analyzed. The protons in the three stereo centra coupled with each other with 8 Hz, indicating *cis* configuration, and 10 Hz, indicating *trans* configuration (Coupling constant values are found in 6.6.1 Synthesis of oseltamivir carboxylate below).

NMR and MS spectra: APPENDIX D on page 106

4.3 CRYSTALLOGRAPHIC STUDIES

The background for this study is that search after oseltamivir phosphate and oseltamivir carboxylate in Cambridge Structural Database gave no hits. The crystal structure of these compounds is therefore not known. In addition to the NMR analysis of oseltamivir carboxylate, the x-ray crystallography can confirm lack of acyl migration and retention of configuration in the stereo centra.

Growth of crystals is a time consuming process and there are several criterion to the crystals morphology before single crystal x-ray crystallography data could be collected. And hence the major limitation to the quality of single-crystal data is crystal quality.

Two different strategies were attempted to produce crystals from both oseltamivir phosphate and oseltamivir carboxylate.

Method 1

Oseltamivir phosphate and oseltamivir carboxylate was dissolved in three different solvents in open vials. The solvent was allowed to evaporate, leaving the solid behind. This method gave a useless amorphous solid which could not be further analyzed by x-ray diffraction.

Table 4 Attempt to grow crystals by method 1

No crystals from this method were obtained.

Solvent	Oseltamivir phosphate	Oseltamivir carboxylate
H_2O	-	-
Methanol	-	-
Ethanol:methanol (1:1)	-	-

Method 2

Oseltamivir phosphate and oseltamivir carboxylate was dissolved in a small amount of water in a tiny tube. The tube was capped and one little hole was made in the sealing. The tube was then placed in a bigger glass, filled with precipitant and capped. The precipitant evaporated

over in the small tube and reduced oseltamivir phosphate and oseltamivir carboxylates solubility, until it eventually began to grow crystals.

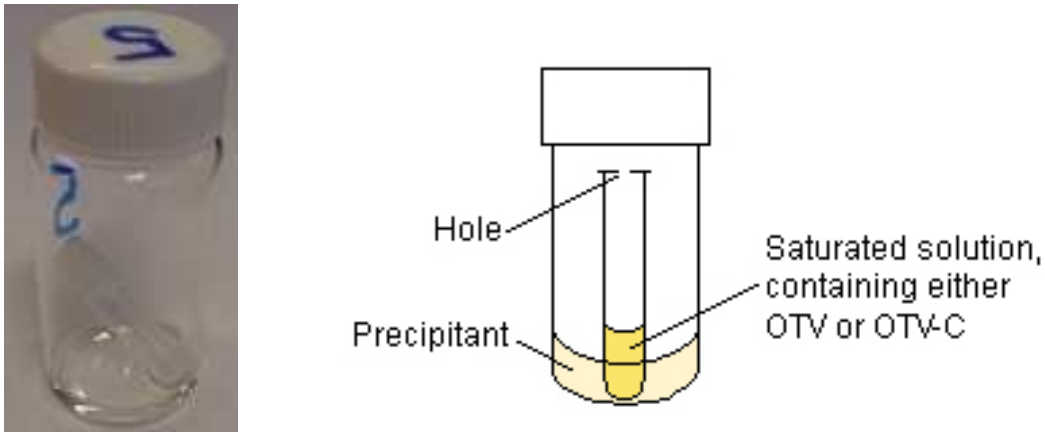


Figure 37 Crystallization of oseltamivir (OTV) phosphate and oseltamivir carboxylate (OTV-C) by method 2

After 2 weeks there was observed crystal growth in two glasses. Oseltamivir phosphate crystals had grown in the glass with acetonitrile as precipitant and oseltamivir carboxylate crystals had grown with acetone as precipitant. These crystals were further studied in the microscope, but their quality was not good enough to obtain x-ray crystallographic data. The crystals grown were needle shaped and the needle grains had connected with each other to form a continuous network. The needles were also very small, less then 10 µm in diameter. The diameter of a good crystal should be approximately 30 µm.

Table 5 Growth of crystals by method 2

Crystal formation was observed in two glasses (indicated with +) after 2 weeks.

Precipitant	Oseltamivir phosphate	Oseltamivir carboxylate
Acetone	-	+
Acetonitrile	+	-
Ethanol	-	-
Isopropanol	-	-

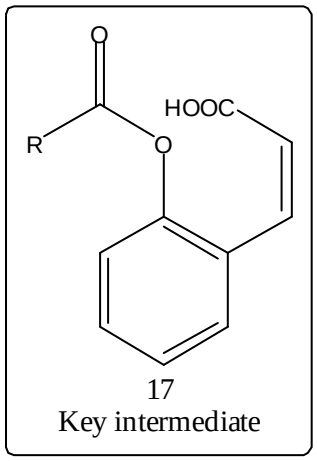
4.4 SYNTHESIS OF THE COUMARIN BASED PRODRUG

To produce a coumarin based prodrug of oseltamivir carboxylate, a key intermediate had to be synthesized (Figure 38).

Three different strategies for synthesizing this key intermediate are described in the literature [**80**].

Strategies:

1) Synthesis from coumarin
2) Photoisomerization of cinnamic acid
3) Catalytical hydrogenation of an alkyne from benzofuran-2-carboxylic acid or 2-iodophenol



17
Key intermediate

Figure 38 The key intermediate in the synthesis of coumarin prodrugs

The compounds in this thesis are numbered after their origin, except for equally structures which are equally numbered. Compounds from strategy 1 are numbered from 11-17, strategy 2 compounds from 21-24 and strategy 3 compounds from 31-37.

4.4.1 Synthesis from coumarin

The first strategy attempted was to synthesis the key intermediate for the commercially available coumarin through a sequence of reactions (6 linear steps). The procedure for this reaction was described by Wang *et al*. (1996) and was used in attempt to synthesize the key intermediate.

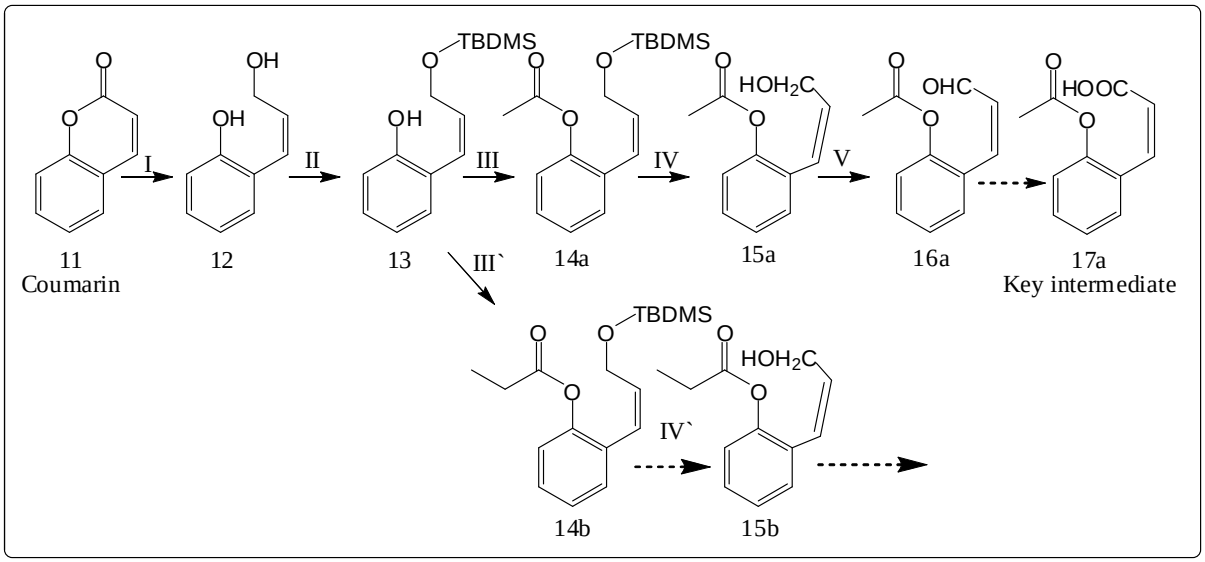


Figure 39 Synthesis from coumarin
(A more detailed scheme can be found in appendix A below.)

4.4.1.1 Reaction I, synthesis of compound 12

The first step in the synthesis was to reduce coumarin to the open ring with a diol by using lithium aluminum hydride (LAH). This synthesis is described in the literature [**63**]. Coumarin was dissolved in ether and LAH was added. After stirring for 15 min on an ice bath, the reaction mixture was acidified with 1 M HCl. The mixture became a dark yellow emulsion. Already at this point something had obviously gone wrong, because the solution was supposed to be extracted with ether. This was not possible and addition of ether just gave a larger amount of emulsion. The acidifying of the solution is an important step, because a longer reaction time than 15 min, would lead to formation of the over reduced product without the exo double bond. This had probably happened, since the pH after the addition on acid still was basic (~ 9-10). More acid was added, but the mixture remained an emulsion. Even though some of the product might have been over reduced, attempts were made to purify the whole emulsion by preparative HPLC after evaporation. This was not successful, and modification of the procedure had to be made.

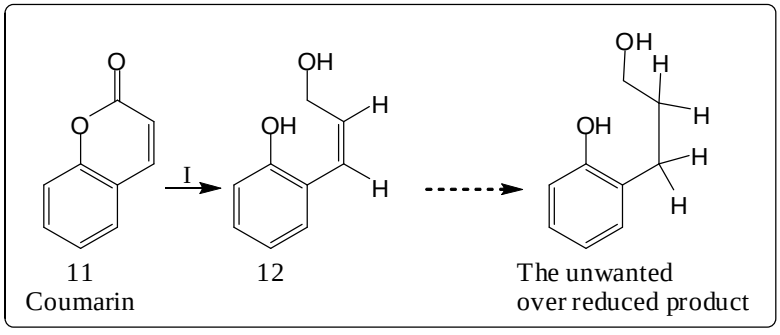


Figure 40 Synthesis of compound 12

Several attempts in accordance with the literature revealed that the acidification step had to be modified. A stronger acid, 5 % HCl instead of 1 M HCl, became the solution. This was attempted since the described amount of acid not was sufficient to acidify the solution. This gave two separated layers, which were extracted with ether. Under the addition of acid, the solution got back its yellow color, which gradually disappeared, until the solution was only light yellowish. This also indicated than the pH of the solution was acid. After extraction of the solution with ether, the combined ether layers was dried and evaporated. The residue was easily dissolved in a small amount of ACN and purified by preparative HPLC.
The mean yield of compound 12 was approximately 44% in all the successful attempts.

4.4.1.2 Reaction II, synthesis of compound 13

Compound 13 was synthesized as described in Wang *et al.* [**63**]. This was a straight forward procedure which stirred for 14 h. 4-Dimethylaminopyridine (DMAP) was added as a catalyst for the reaction (Figure 41 below). *tert*-Butyl-dimethylsilyl (TBDMS) chloride was used as protective group for the primary hydroxyl group. The TBDMS attached selectively to the primary hydroxyl group by controlling the reaction time, more than 14h stirring would give the side product with TBDMS attached to both the primary and the phenol hydroxyl group. The mean yield of compound 13 in all the studies done was ~ 45 %.

This yield was some lower than described in the literature (83 %). One difference from the literature procedure that might have affected the yield of the reaction was the temperature. The reaction mixture was placed on an ice bath to keep the temperature at 0 °C and stirred over the night. The temperature was not kept at 0 °C for all the 14 hours, because the ice gradually melted and the temperature of the reaction rose. This might be one of the reasons for the lower yield in this reaction.

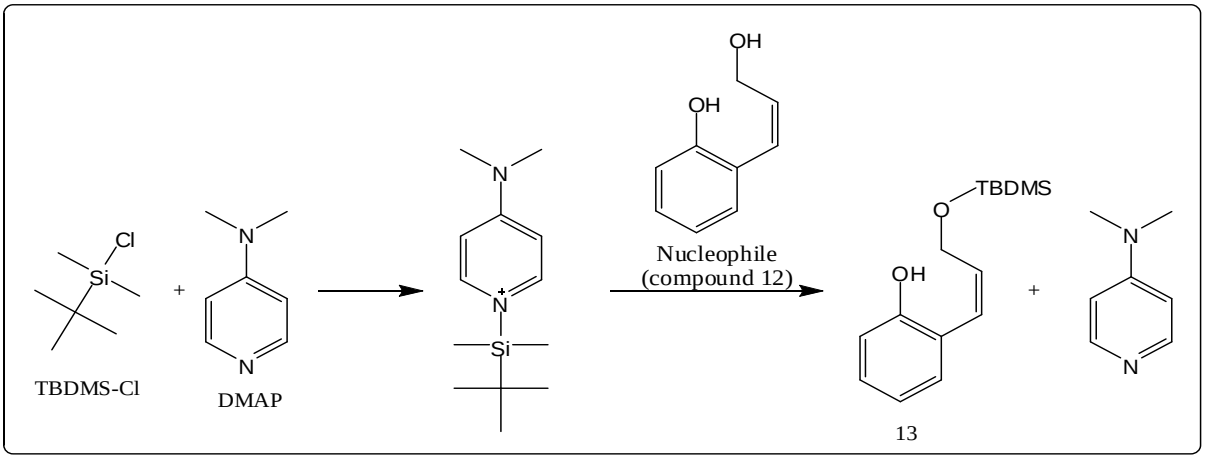


Figure 41 Mechanism for DMAP as catalyst

4.4.1.3 Reaction III and III', synthesis of compound 14a and 14b

Compound 14a and 14b was synthesized in similar manners, both as described in Wang *et al.*, and with acetic anhydride and propionic anhydride as reagent, respectively. DMAP and triethylamine (TEA) was added as catalysts. In the literature procedure (Wang *et al*.), the reaction stirred for 1 h under N_2 atmosphere. In process analysis of the reaction mixture on analytical HPLC revealed that 15 minutes was a sufficient reaction time.
The mean yield for reaction III was ~ 58 % and for reaction III' ~ 69 %.
The compounds were purified by preparative HPLC with an acid free eluent, to avoid cleavage of the protection group and the ester.

4.4.1.4 Reaction IV and IV', synthesis of compound 15a and 15b

In reaction IV and IV' the TBDMS protective group of the primary hydroxyl was cleaved. This was easily done under acid conditions, because TBDMS is relatively sensitive to acid [**81**]. In the literature the reaction stirred for 3h. This time was shortened, because after 3h the ester in the molecule also got cleaved. By in process HPLC analysis of the reaction, 1h revealed to be a sufficient reaction time. The mean yield for reaction 15a was 84 %.
The yield of compound 15b after purification was approximately 0 %, because of the very small amount of starting material. But results form HPLC analysis confirmed that 15b most likely had been synthesized. No further attempts were made to synthesize 15b, because all resources were used to optimize the synthesis of compound 15a to compound 17a.

4.4.1.5 Reaction V, synthesis of compound 16a

In reaction V compound 15a was oxidized to compound 16a by pyridinium chlorochromate (PCC). PCC was first described by Corey *et al.* and it was developed especially for the oxidation of primary alcohols to aldehyde [**82**]. The reaction was preformed under dry condition as described in the literature [**63**]. The byproduct (Cr^{3+}) of the reaction deposits with pyridine and gave a sticky black tar. This reaction was only synthesized successfully once, at the first attempt. Because only ^{1}H-NMR analyses was preformed on the first reaction, and later attempts were unsuccessful, this compound lacks ^{13}C-NMR data.
This product might also have oxidize further to the carboxylic acid under the preparative purification process because present oxygen. Analysis for the carboxylic acid was not done.

4.4.2 Photoisomerization of *trans*-2-hydroxycinnamic acid

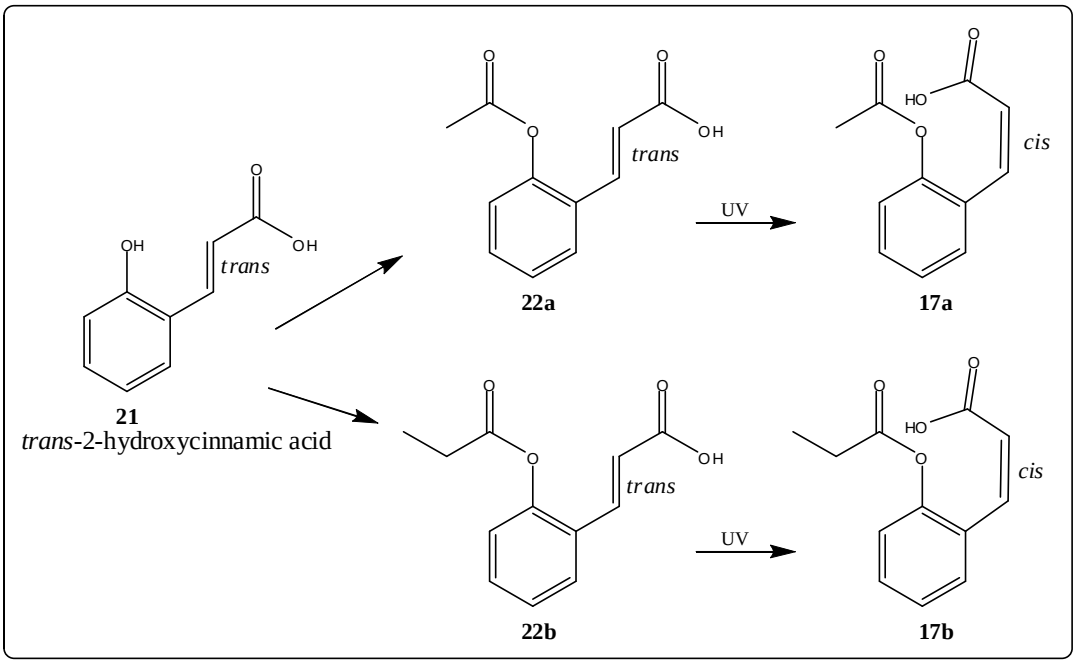


Figure 42 Synthesis of the key intermediate (compound 17) by using a photochemical approach

The photochemical isomerization of *trans* cinnamic acid have received considerable attention, and for almost 100 years ago there was observed formation of small amounts of the *cis*-isomer in experiments were *trans* cinnamic acid was exposed to sunlight [**83**]. This photochemical approach takes advantage of the known *trans* to *cis* photoisomerization of cinnamic acid. The cinnamic acid was photolyzed at 365 nm, because studies have shown that long UV wavelength favores the *trans* to *cis* isomerization and shorter wavelengths favores the *cis* to *trans* isomerization [**80, 84, 85**]. Generally, the *cis* alkene is less stable than their *trans* isomer, because of steric hindrance.

4.4.2.1 Synthesis of compound 22a and 22b

Compound 22a and 22b was synthesized as described in Zheng *et al*. [**80**]. This was a straight forward synthesis with an high overall yield. TEA and DMAP were added as catalysts. After stirring for 2h, the reaction mixture was washed with 1M HCl, and this removed both DMAP and TEA. The mean yield of compound 22a and 22b was approximately 95 % in all experiments.

Compound 22a is a commercial available chemical. This compound was rather synthesized than bought because:

- the synthesis from compound 21 was straight forward with a very high yield
- compound 21 was available in the laboratory and cheaper

4.4.2.2 Synthesis of compound 17a and 17b by photoisomerization

Compound 22 was dissolved in methanol and placed under two UV lamps for irradiation at 365 nm. In Zheng *et al*., 24h of irradiation gave an yield of approximately 75 % for compound 17a [**80**]. In this thesis experiments a much longer irradiation time was necessary. After 48h only ~ 9 % of the *trans* isomer was converted to *cis*, based on ^{1}H NMR analysis. The *cis* isomer was isolated by preparative HPLC.

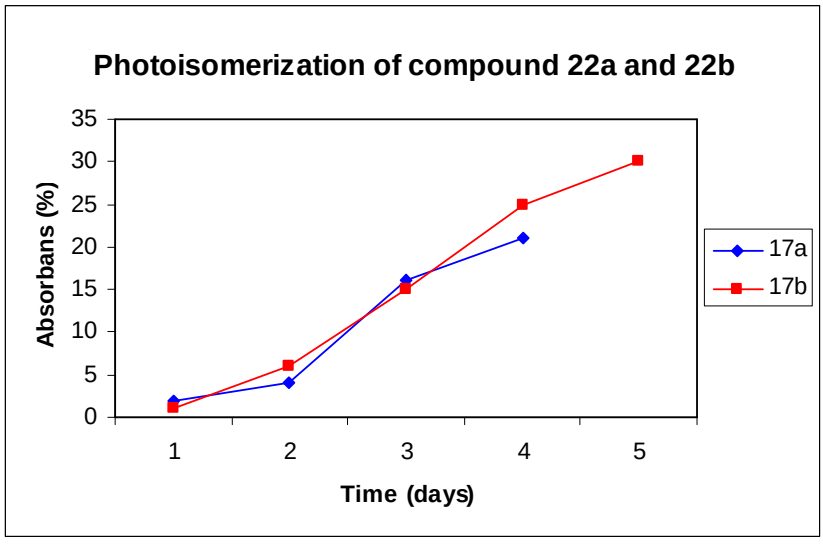
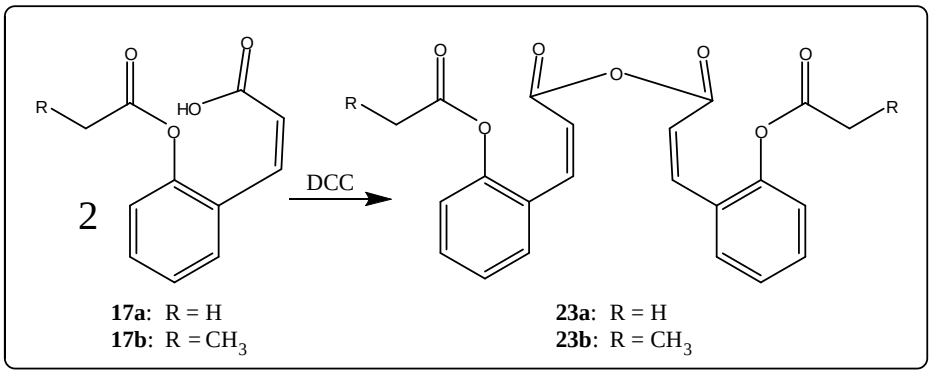


Figure 43 Photoisomerization of compound 22a and 22b to 17a and 17b, respectively[6].

[6] Diagram based on results from HPLC analysis.
Absorbans measured at 250 nm.

The figure above indicates that the photoisomerization of compound 22 is a time consuming process. And several days was necessary to yield a satisfaction conversation of the *trans* isomer. The literature described a much higher yield and a much shorter reaction time for this synthesis. The reason for this must be the equipment. The UV-lamps that were used in this thesis experiments was not designed for photochemistry, which Zheng *et al.* most likely had. The power level of the UV-lamp is also of importance. Wang *et al.* have described that low power UV lamps (4 W) are favored over higher powered, e.g. 500 W, UV lamps [**84**]. In this thesis it was used two low powered hand held UV lamps, one with 6 W and one with 12 W power. Higher powered lamps were associated with potential photochemical side reactions. Many unknown factors may have influenced this reaction, but most of them must relate to the equipment. The hand held UV lamps used were not designed for photochemistry, but for TLC plate viewing, fluorochemistry, food inspection, quality control, titration, pesticide analysis and mineralogy [**86**]. Special equipment, e.g. glassware and apparatus, for photochemical reactions can be bought from commercial institutions, but due to the limited time this was not further investigated or bought.

4.4.2.3 Attempted synthesis of compound 23a and 23b



The synthesis of acid anhydride was attempted with use of 0.5 eq dicyclohexylcarbodiimide (DCC). Synthesis of acyclic anhydride are known to be difficult to prepare directly from the corresponding acids [**87**]. But use of DCC as couplings agent have been used successfully by others on the Department of Pharmaceutical Chemistry, so it was made an attempt to make this acid anhydride. This synthesis was not successful, the ^{1}H NMR and ^{13}C NMR analysis showed no signal from the product. This might be because this compound has sp^2 configuration α to the carbonyl, which also is conjugated with a benzene ring and this might complicate the synthesis.

4.4.2.4 Attempted synthesis of compound 24b

Acid chlorides are more reactive than acid anhydrides and are relatively easy to prepare. The carboxylic acid was therefore converted into an acid chloride by treatment with oxalyl chloride, other agents, like thionyl choride, could also have been used. A catalytic amount of dimethylformamide (DMF) was added and the reaction was monitored by HPLC (G1) analysis.

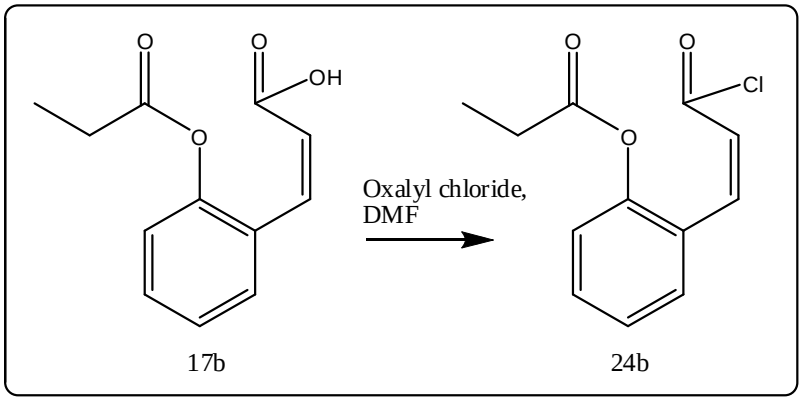


Figure 44 Synthesis of the acid chloride 24b

Since acid chlorides are too reactive to be monitored itself, the formation of the methyl ester of compound 24b was observed instead. 1 droplet of the reaction mixture was added 1 ml methanol in an HPLC vial. The acid chloride reacted immediately with methanol to give the methyl ester of compound 24b (Figure 45). To ensure that all acid chloride molecules had reacted, a small amount of water was also added. The observation of methyl ester based on the principle that the methyl ester of compound 24b had a longer retention time than compound 17b, 9.0 minutes compared to 7.1 minutes respectively, and could therefore be used as an indicator for the formation of acid chloride.

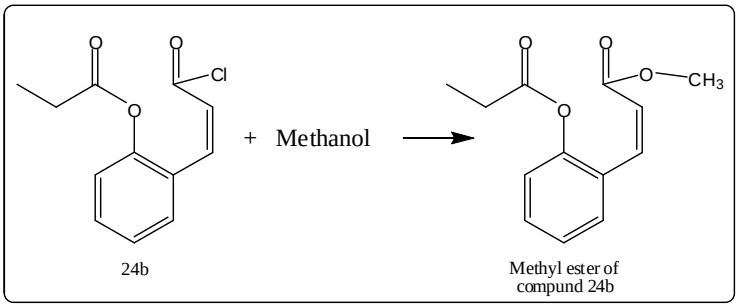


Figure 45 The synthesis of acid chloride was monitored by HPLC analysis of the methyl ester of compound 24b

The HPLC analysis indicated that the acid chloride was synthesized. Because a surplus of oxalyl chloride had been used and this reaction generates 1 molecule of acid (HCl), the

reaction was to acid for the ester in the product, which decomposed under the careful evaporation.

Due to small amounts of the reactant, long reaction time of the photoisomerization and lack of time, attempts to optimize this procedure were not done in this thesis.

4.5 ALTERNATIVE STRATEGIES NOT ATTEMPTED

4.5.1 Catalytical hydrogenation of an alkyne

This strategy was not attempted in practice, but is mention as an alternative to the two other strategies above. The strategy takes advantage of known literature procedures to convert benzofuran-2-carboxylic acid (compound 31) or 2-iodophenol (compound 32) into 3-(2-hydroxyphenyl)propynoic acid (compound 33) as described in Figure 46 [**80, 88, 89, 90, 91**].

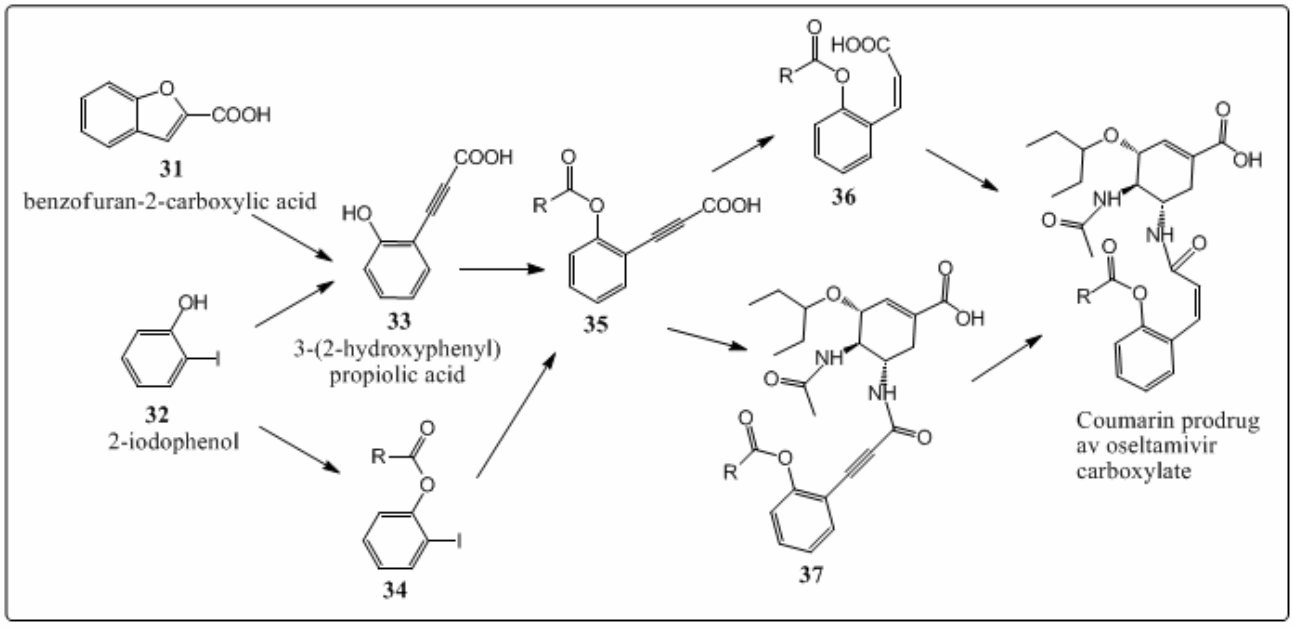


Figure 46 The catalytical hydrogenation approach

Synthesis from compound 31 [**80**]

Compound 33 can be synthesized from compound 31 as described in Zheng *et al.* After purification this reaction had an 89 % yield. Compound 33 is further treated with acid anhydride, to give compound 35 with above 80 % yield. Compound 35 can either be hydrogenated in the presence of Lindlar catalyst and purified to give compound 36 (yield 77 %) before it is coupled with oseltamivir carboxylate or the coupling with oseltamivir

carboxylate can be synthesized first, compound 37, and then hydrogenated to yield the coumarin prodrug. Each step will be purified by preparative HPLC.

Synthesis from compound 32

The synthesis of compound 33 and 35 from compound 32 and 34, respectively, is achieved through a sonogashira reaction. This can be preformed by a similar synthesis strategy as described in Hadfield *et al*. or Pal *et al.* [**89, 91**]. Compound 34 is synthesized by treating compound 32 with an acid anhydride before the sonogashira reaction.
Compound 35 is treated in the same matter as described above for the syntesis from compound 31. Each compound will be purified by preparative HPLC before further synthesis.

4.5.2 Synthesis of the cinnamic acid succinimido ester

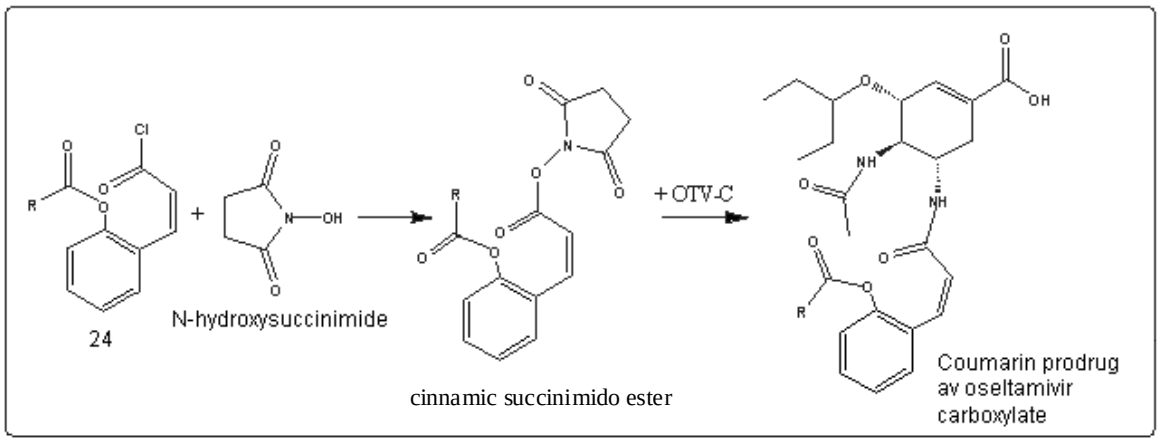


Figure 47 Alternative approach to synthesize the coumarin prodrug

This approach is an additional alternative for the coupling of acid chloride with the oseltamivir carboxylate. Compound 24 is first coupled with N-hydroxysuccinimide in a nonpolar solvent, add triethylamine as catalyst. Then the literature procedure described in McCafferty *et al* is used for coupling of oseltamivir carboxylate to this cinnamic succinimido ester [**92**]. In this procedure trichloroethaol must be used instead of DMF as solvent because of the solubility problem with oseltamivir carboxylate in non polar solvents.

Table 6 Solubility of oseltamivir carboxylate

Milligram quantities of oseltamivir carboxylate was dissolved in 1 ml solvent and stirred in the ultrasound bath.

Solvent	Dissolved?
Acetone	No
ACN	No
ACN:DCM (1:1)	No
ACN:THF (1:1)	No
Chloroform	No
DCM	No
DMF	No
DMSO	No
H_2O	Yes
Methanol	Yes
Pyridine	Yes
Tetrahydrofurane	No
Trifluorethanol	Yes

5 CONCLUDING REMARKS

The hydrolysis of oseltamivir phosphate to its active metabolite oseltamivir carboxylate was studied under different acid condition. These studies indicated that hydrolysis in 25 % HCl for 1 day gave the best yield of oseltamivir carboxylate.

The aim in this thesis was to study the synthesis around some novel prodrugs of oseltamivir carboxylate with potential higher protein binding to albumin then its parent compound oseltamivir carboxylate. Coumarin was chosen as pro moiety for these compounds. The major advantage with coumarin is that it has been studied for many years and the toxicity profile is therefore well known. These prodrugs should contain a free carboxyl acid which binds to albumin and ensures that the overall molecule is not to hydrophobic. According to Lipinski *et al.* a drug would have poor absorption and penetration if the molecule has more then five hydrogen binding donors, molecule weight over 500, log P over 5 and has more then 10 hydrogen binding acceptors [**77**].

The coumarin prodrugs are based on the pro prodrug concept. The pro pro moiety is cleaved by esterase *in vivo* and thereby the amid bound prodrug takes advantage of the facile lactonization of cinnamic acid which restore the parent drug and a nontoxic coumarin moiety. The coumarin prodrugs have to be tested in many different assays to clarify its kinetic and targeting effect. The obviously advantage to synthesize these prodrugs is that they might have higher protein binding then oseltamivir which has only an ethyl ester as pro moiety.

Two different strategies were attempted to synthesis the key intermediate for the coumarin prodrug of oseltamivir carboxylate. The first strategy was to make the prodrug moieties from the cheap and commercially available coumarin and the second strategy was to synthesis the key intermediate trough a photochemical approach.

The synthesis from coumarin was chosen for the first attempt, but because this synthesis required a long sequence of reactions (6 linear steps) with a low overall yield, the key intermediate became difficult to develop. Even though several attempts were made, improved synthesis and larger scale, the final key intermediate was not synthesized from this strategy. The photochemical approach gave the key intermediate, but not in time and not in sufficient amount to do a successful coupling to oseltamivir carboxylate.

Two alternative synthesis strategies have been suggested to synthesize the key intermediate and to optimize the coupling of the promoiety to oseltamivir carboxylate. If there had been more time these approaches would have been attempted. There were also further plans for testing of the synthesized coumarin prodrug if they had been synthesized.

The coumarin prodrugs are design to have a higher protein binding to human serum albumin than oseltamivir and avoid complete hydrolysis by first pass metabolism. The plan was to study different prodrugs in assays with human serum, were the combined effect of protein binding and the breakdown profile by esterase is observed. The breakdown profile by different esterases, for example hepatic esterases, is also an interesting approach. In addition, possible binding sites for these prodrugs on albumin could also have been investigated. Animal studies might be preformed to investigate the targeting effect of these new prodrugs.

6 EXPERIMENTAL

6.1 MATERIALS AND METHODS

6.1.1 Reagents

Water: Elga PureLab Maxima HPLC
(ion exchanged, HPLC-grade water)

Other reagents: APPENDIX E

6.1.2 Solvents

6.1.2.1 Solvents for synthesis

Refers to APPENDIX E

6.1.2.2 Solvents for HPLC-analysis

HPLC-grade quality from commercial institutions (Fluka, Sigma-Aldrich, Merck, Kebo-Lab, Riedel-de Haën).

6.1.2.3 Deuterated solvents

Chloroform-d (CDCl3), 99.8 atom % D:	Aldrich
(methylsulfoxide)-d6 (DMSO-d6), “100” (min. 99.96 atom % D):	Aldrich
Methanol-d4 (CD_3OD-d4), 99.8 atom % D:	Aldrich
Deuterium oxide-d2 (D_2O), 100 atom % D:	Aldrich

6.1.3 Solutions

6.1.3.1 Formic acid solution pH 3

0.46 g of formic acid was diluted to 1 liter with purified water, giving a concentration of 10 mM.

6.1.4 Solvents for NMR

^{1}H and ^{13}C NMR were recorded on a Bruker Avance DPX 300 instrument, at 300 MHz and 75 MHz respectively. All experiments were performed at 25 °C with deuterated solvents.

Reference peaks [**93**]:	$CDCl_3$:	^{1}H δ 7.25	^{13}C δ 77.0
	DMSO-d6:	^{1}H δ 2.5	^{13}C δ 39.7
	MeOH-d4:	^{1}H δ 3.35	^{13}C δ 49.0

6.2 HPLC ANALYSIS

6.2.1 Apparatus

Technical details

Autosampler:	HP G1313A
Pump:	HP Aglient 1100 series binary pump
Coloumn:	C18 250 x 2 mm, 5 µ particle size, Luna, Phenomenex
Detector:	HP G1315A Diode Array Detector
Data acquisition and handling:	HP Chemstation version A.06.03 [509]

Experimental parameters

Coloumn temperature:	Ambient
Injection volume:	5 µl
Flow rate:	0.5 ml/min
Mobile phase:	Gradient and isocratic
Chromatogram run time:	14 min and 20 min
Detection wavelengths:	UV, 317 nm, 210 nm and 250 nm, bond with 16

6.2.2 Mobile phase

Gradient 1 (G1)

Time (min)	Formic acid 10 mM, pH 3 (%)	ACN (%)	Flow (ml/min)
0	80	20	0.5
8	20	80	0.5
12	20	80	0.5
14	80	20	0.5
-	80	20	0.5

Isocratic 1 (I1)

Time (min)	H_2O (%)	ACN (%)	Flow (ml/min)
-	90	10	0.5

This isocratic eluent was used for analyzing the hydrolysis of oseltamivir phosphate.

6.3 PREPARATIVE HPLC

Technical details

Injector:	Manual
Pump:	Modified HP 1050
Coloumn:	250 x 20 mm packed with LiChropre® RP-18, 25-40 µm particles
Detector:	Labomatic Labcord-200 UV spectrophotometer
Data acquisition and handling:	Absorbance was read from the detector while chromatographying

Experimental parameters

Coloumn temperature:	Ambient
Injection volume:	5-10 ml
Flow rate:	10 ml/min
Mobile phase:	Isocratic
Detection wavelength:	UV, 220 nm and 250 nm

6.3.1 Mobile phase

Isocratic mobile phases were prepared using different volume ratios of ACN and formic acid-solution pH 3 (HCOOH (aq)) or H_2O. Choice of ratio was based on analytical HPLC; the isocratic eluent that gave retention times of about 6-8 minutes was chosen as the mobile phase.

6.4 UV LAMPS

6.4.1 UV lamp 1

Technical details

Producer:	UVItec Limited
Model:	LF 106L
Wavelength:	365 nm
Power:	12 W
Frequency:	50 Hz
Voltage:	230 V

6.4.2 UV lamp 2

Technical details

Producer:	Vilber Lourmat
Model:	VL-6.L
Wavelength:	365 nm
Power:	6 W
Frequency:	50 Hz
Voltage:	230 V

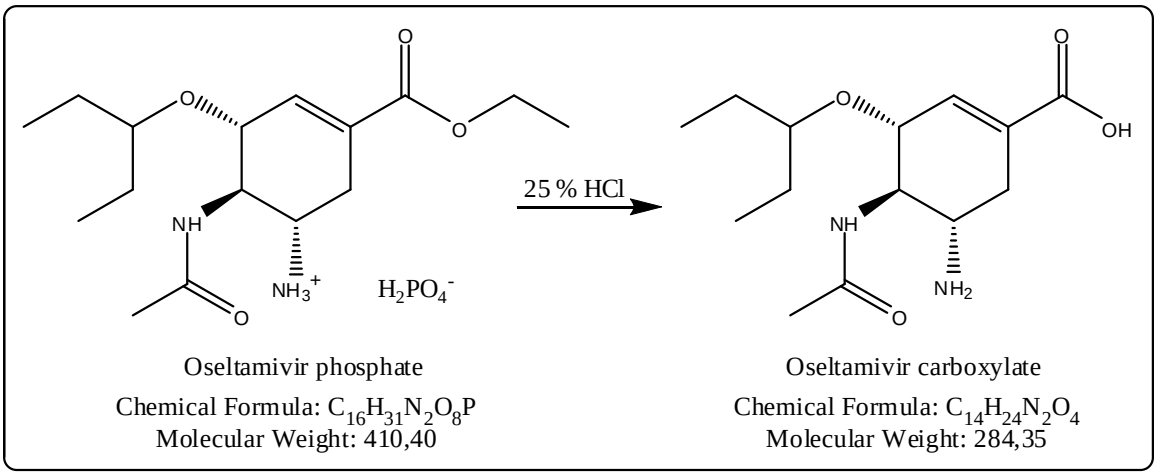
6.5 MICROSCOPY

Technical details

Producer:	Zeiss
Model:	SteREO Discovery.V12

6.6 SYNTHESIS

6.6.1 Synthesis of oseltamivir carboxylate



Oseltamivir phosphate
Chemical Formula: $C_{16}H_{31}N_2O_8P$
Molecular Weight: 410,40

Oseltamivir carboxylate
Chemical Formula: $C_{14}H_{24}N_2O_4$
Molecular Weight: 284,35

Oseltamivir phosphate (800 mg, 1.95 mmol) was dissolved in HCl (1 ml, 25 %). The solution stirred at room temperature over night. After neutralization to pH 7 by Na_2CO_3(s), the solution was purified by preparative HPLC with ACN/H_2O (10:90) to afford oseltamivir carboxylate as a white solid.

Yield: 302 mg (54.1 %)

HPLC: R_t: 4.6 minutes (I1)

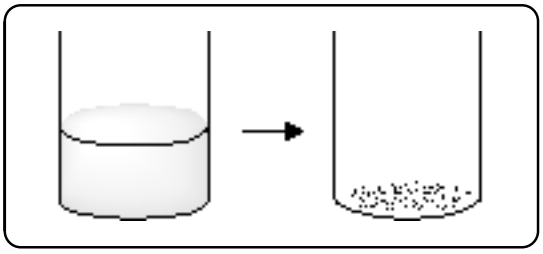
^{1}H NMR (300 MHz, CD_3OD): δ 0.91 (q, 6H, J=7.4Hz), 1.53 (m, 4H), 2.03 (s, 3H), 2.42 (ddt, 1H, J=2.8Hz, J=10.0Hz, J=17.4Hz), 2.86 (dd, 1H, J=5.5Hz, J=17.4Hz), 3.41 (m, 2H), 3.94 (dd, 1H, J=8.2Hz, J=11.0Hz), 4.15 (d, 1H, J=8.2Hz), 6.59 (t, 1H, J=2.0Hz)

^{13}C NMR (75 MHz, CD_3OD): 9.650, 9.878, 23.207, 26.667, 27.308, 31.138, 51.421, 54.881, 76.413, 83.380, 133.337, 134.596, 173.696, 174.651

MS (ES^+; TOF): m/z 197.0 (9 %), 285.1 ($[M+H]^+$ = 100 %)

6.6.2 Growth of oseltamivir phosphate crystals

Method 1



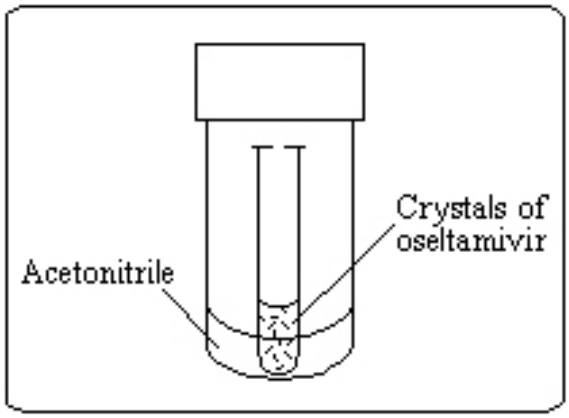
Oseltamivir phosphate (20 mg) was weight out in three small glasses. The three glasses was added water (100 µl), methanol (200 µl) and methanol:ethanol (1:1) (1.2 ml), respectively. The solution was allowed to evaporate at room temperature over night.

The solid obtained after the solvent had evaporated was observed in the microscope.
No crystals were observed.

Method 2

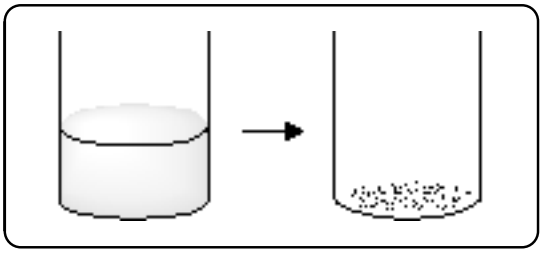
Oseltamivir phosphate (approximately 0.5-2 mg) was weight out in four small tubes and dissolved in water (30 µl). The tubes were capped and a little hole was made in the sealing. The tubes were then placed in four glasses. Each of the glasses was added precipitant, acetone (1 ml), acetonitrile (1 ml), ethanol (1 ml) and isopropanol (1 ml), respectively and closed.

After two weeks, crystal growth were observed in the glass with acetonitrile as precipitant. The crystals were observed in the microscope. The quality of the crystal was not good enough for further x-ray study.



6.6.3 Growth of oseltamivir carboxylate crystals

Method 1



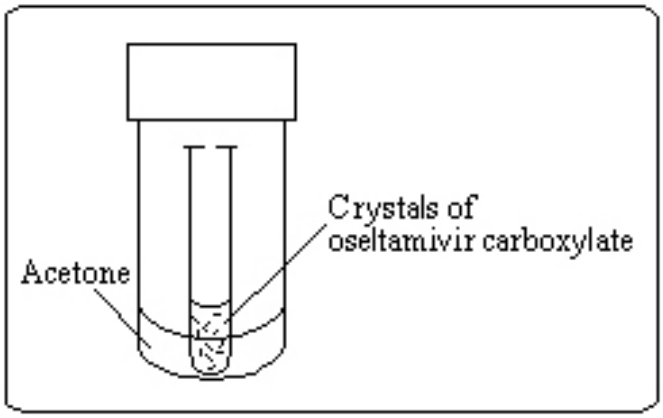
Oseltamivir carboxylate (20 mg) was weight out in three small glasses. The three glasses was added water (100 µl), methanol (200 µl) and methanol:ethanol (1:1) (600 µl), respectively. The solution was allowed to evaporate at room temperature over night.

The solid obtained after the solvent had evaporated was observed in the microscope.
No crystals were observed.

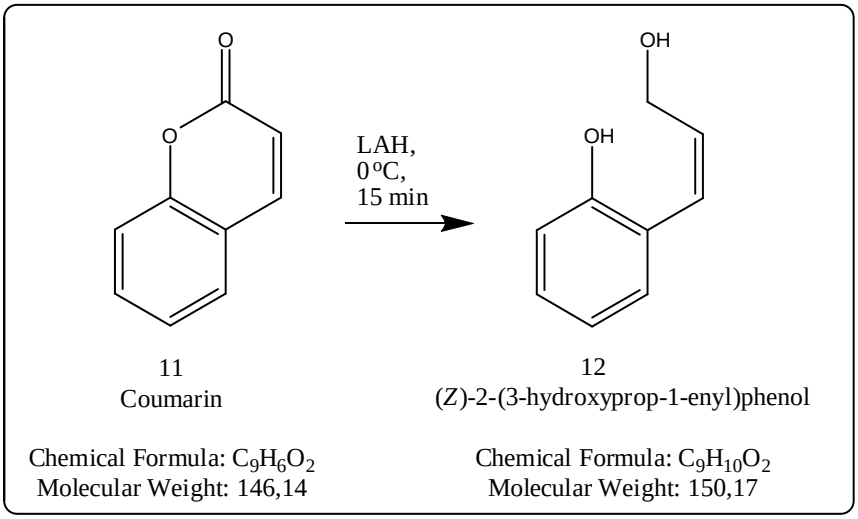
Method 2

Oseltamivir carboxylate (approximately 0.5-2 mg) was weight out in four small tubes and dissolved in water (30 µl). The tubes were capped and a little hole was made in the sealing. The tubes were then placed in four glasses. Each of the glasses was added precipitant, acetone (1 ml), acetonitrile (1 ml), ethanol (1 ml) and isopropanol (1 ml), respectively and closed.

After two weeks, crystal growth were observed in the glass with acetone as precipitant. The crystals were observed in the microscope. The quality of the crystals was not good enough for further x-ray study.



6.6.4 Synthesis of compound 12, (Z)-2-(3-hydroxyprop-1-enyl) phenol



11
Coumarin

Chemical Formula: $C_9H_6O_2$
Molecular Weight: 146,14

12
(Z)-2-(3-hydroxyprop-1-enyl)phenol

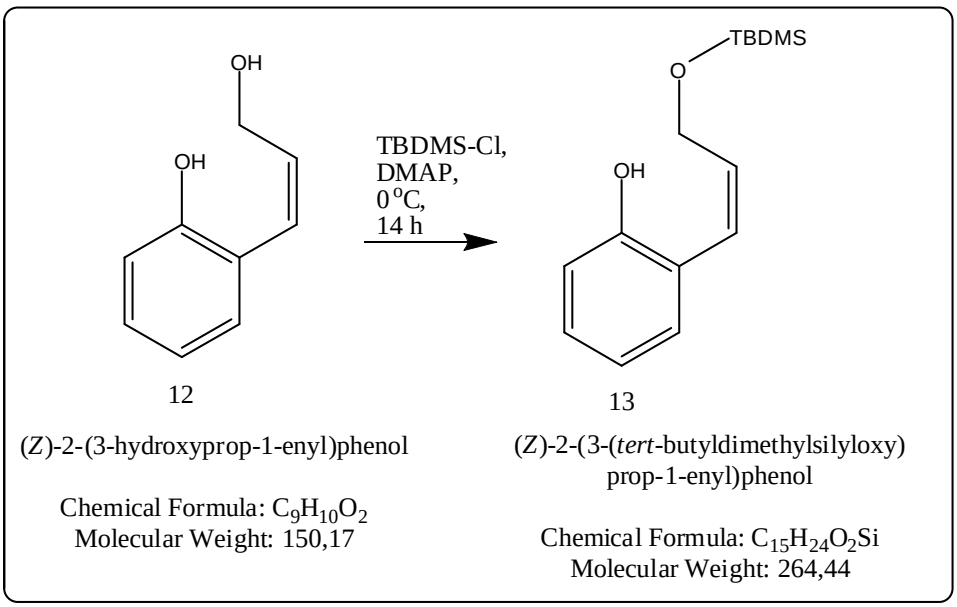
Chemical Formula: $C_9H_{10}O_2$
Molecular Weight: 150,17

Coumarin (3.65 g, 25 mmol) was dissolved in ether (100 ml) and placed in a cold bath. Then the solution was treated at 0 °C with a solution of lithium aluminum hydride (LAH) 1.0 M in ether (50 ml, 50 mmol). After stirring for 15 min, 5 % HCl (25 ml) was added to the reaction at 0 °C. Then the solution was adjusted to pH 5 with 1M HCl and extracted with ether (3x75 ml). The ether layer was dried over Na_2SO_4. The drying agent was removed by gravity filtration and the filtrate evaporated. The residue was then re-dissolved in ACN and purified by preparative HPLC with ACN/HCOOH (aq) (25:75) to afford compound 12 ((Z)-2-(3-hydroxyprop-1-enyl)phenol) as a white solid.

YIELD:	2.41 g (64.3 %)
HPLC:	R_t: 4.6 minutes (G1)
^{1}H NMR (300 MHz, CD_3OD):	δ 4.26 (dd, 2H, J=6.5Hz), 5.80 (m, 1H), 6.63 (d, 1H, J=11.7Hz), 6.79 (m, 2H), 7.08 (m, 2H)
^{13}C NMR (75 MHz, CD_3OD):	δ 60.19, 116.23, 120.11, 125.07, 127.28, 129.72, 131.29, 131.78, 156.10 There were some overlapping signals.

6.6.5 Synthesis of compound 13,

(Z)-2-(3-(*tert*-butyldimethylsilyloxy)prop-1-enyl)phenol [63]



12

(*Z*)-2-(3-hydroxyprop-1-enyl)phenol

Chemical Formula: $C_9H_{10}O_2$
Molecular Weight: 150,17

13

(*Z*)-2-(3-(*tert*-butyldimethylsilyloxy) prop-1-enyl)phenol

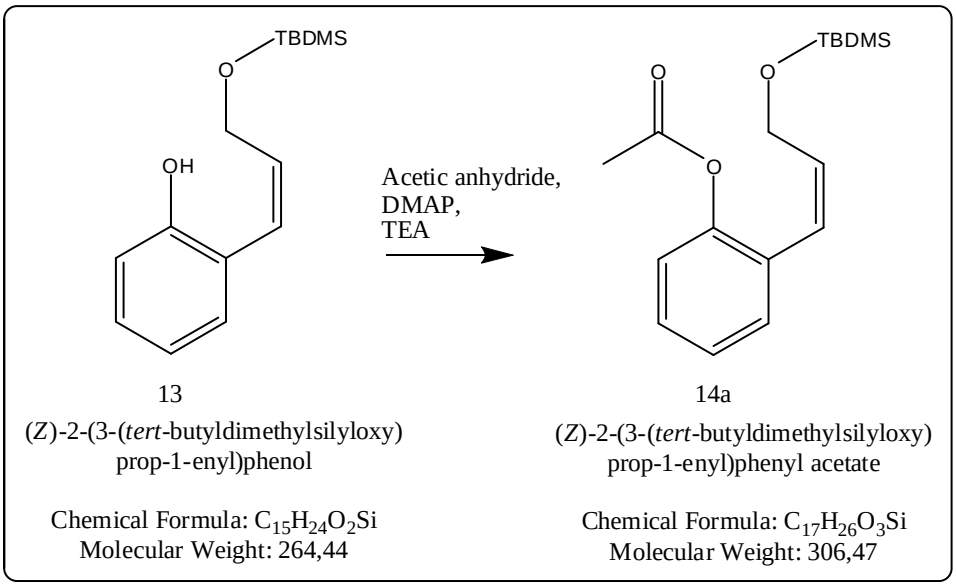
Chemical Formula: $C_{15}H_{24}O_2Si$
Molecular Weight: 264,44

A solution of compound 12 (3.425 g, 22.8 mmol) was dissolved in dry THF (35.5 ml) and placed in a cold bath. To the stirred solution *tert*-butyl-dimethylsilyl (TBDMS) chloride (3.787g, 25.1 mmol) was added at 0 °C. Then N,N-dimethylpyridin-2-amine (DMAP) (4.182 g, 34.2 mmol) in dry THF (44 ml) was drop wise added. After stirring for 14 h at 0 °C, the solution was filtered and evaporated to remove the THF. The residue was re-dissolved in ethyl acetate (44 ml) and washed with 1 M HCl (2 x 27 ml), 5 % $NaHCO_3$ (22 ml) and H_2O (22 ml). The ethyl acetate layer was dried over Na_2SO_4, filtered, and evaporated. The residue was then re-dissolved in ACN and purified by preparative HPLC with ACN/H_2O (70:30) to afford compound 13 ((Z)-2-(3-(*tert*-butyldimethylsilyloxy)prop-1-enyl)phenol) as a light yellow solid.

YIELD:	3.485 g (57.8 %)
HPLC:	R_t: 13.3 minutes (G1)
^{1}H NMR (300 MHz, CD_3OD):	δ 0.03 (s, 6H), 0.88 (s, 9H), 4.36 (dd, 2H, J=6.3Hz), 5.77 (dt, 1H, J=6.3Hz, J=11.8Hz), 6.61 (d, 1H, J=11.7Hz), 6.77-7.07 (m, 4H)
^{13}C NMR (75 MHz, CD_3OD):	δ -4.99, 19.16, 26.43, 61.72, 116.24, 120.04, 125.05, 126.95, 129.73, 131.31, 132.05, 156.10 There were some overlapping signals.

6.6.6 Synthesis of compound 14a,

(Z)-2-(3-(*tert*-butyldimethylsilyloxy)prop-1-enyl)phenyl acetate [63]

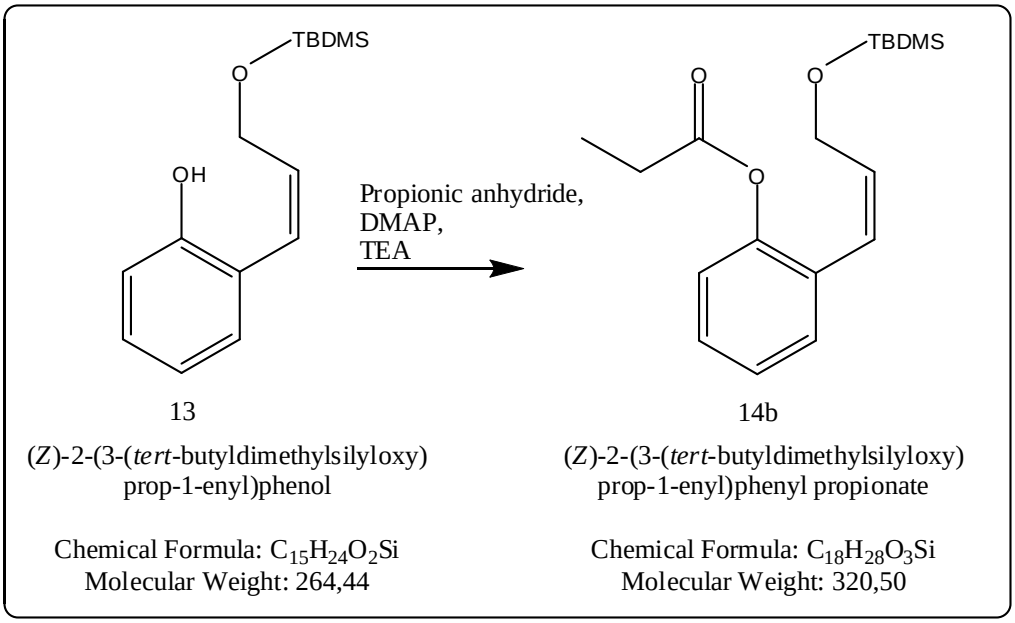


To a solution of compound 13 (3.521g, 13.3 mmol) in dry CH_2Cl_2 (73 ml) was added drop wise acetic anhydride (1.5 ml, 15.8 mmol), then DMAP (325 mg, 2.7 mmol) and triethylamine (TEA) (3.3 ml, 23.8 mmol). After stirring at room temperature under Ar atmosphere for 15 minutes, the reaction mixture was washed with 1 M HCl (2 x 49 ml), 5 % $NaHCO_3$ (37 ml) and H_2O (37 ml). The dichloromethane layer was dried over Na_2SO_4, filtered, and evaporated. The residue was re-dissolved in ACN and purified by preparative HPLC with ACN/H_2O (70:30) to afford compound 14a ((Z)-2-(3-(*tert*-butyldimethylsilyloxy)prop-1-enyl)phenyl acetate) as a white powder.

YIELD:	2.431 g (59.6 %)
HPLC:	R_t: 15.9 minutes (G1)
^{1}H NMR (300 MHz, CD_3OD):	δ 0.01 (s, 6H), 0.87 (s, 9H), 2.26 (s, 3H), 4.29 (dd, 2H, J=1.6Hz, J=6.3Hz), 5.88 (dt, 1H, J=6.3Hz, J=11.7Hz), 6.40 (dt, 1H, J=1.5Hz, J=11.6Hz), 7.08 (m, 1H), 7.24 (m, 2H), 7.33 (m, 1H)
^{13}C NMR (75 MHz, CD_3OD):	δ -5.05, 19.09, 20.81, 26.37, 61.39, 123.46, 125.37, 126.89, 129.80, 131.35, 134.71, 149.76, 171.19 There were some overlapping signals.

6.6.7 Synthesis of compound 14b,

(Z)-2-(3-(tert-butyldimethylsilyloxy)prop-1-enyl)phenyl propionate [63]



13

(Z)-2-(3-(*tert*-butyldimethylsilyloxy) prop-1-enyl)phenol

Chemical Formula: $C_{15}H_{24}O_2Si$
Molecular Weight: 264,44

14b

(Z)-2-(3-(*tert*-butyldimethylsilyloxy) prop-1-enyl)phenyl propionate

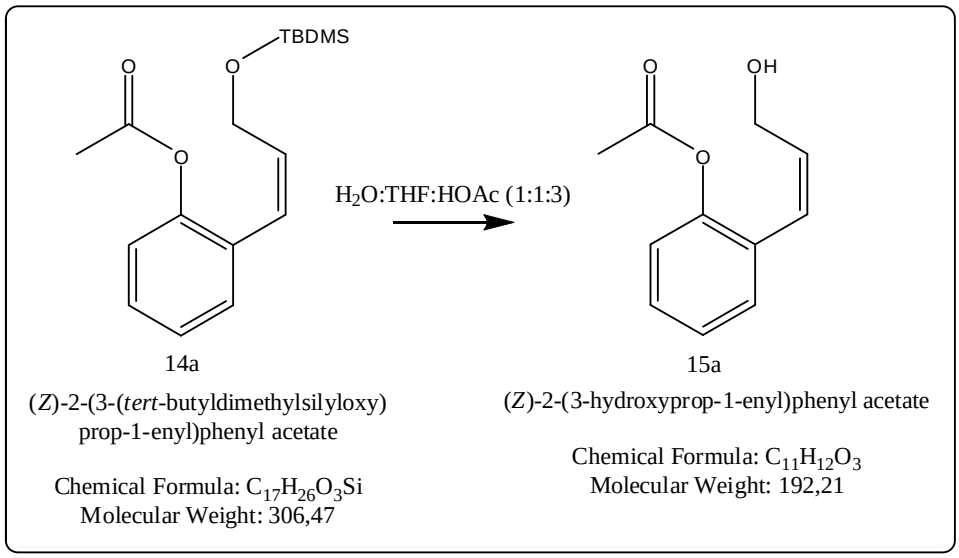
Chemical Formula: $C_{18}H_{28}O_3Si$
Molecular Weight: 320,50

To a solution of compound 13 (526 mg, 1.99 mmol) in dry CH_2Cl_2 (11 ml) was added drop wise propionic anhydride (0.305 ml, 2.34 mmol), then DMAP (48 mg, 0.39 mmol) and TEA (0.5 ml, 3.6 mmol). After stirring at room temperature under Ar atmosphere for 15 minutes, the reaction mixture was washed with 1 M HCl (2 x 9 ml), 5 % $NaHCO_3$ (7 ml) and H_2O (7 ml). The dichloromethane layer was dried over Na_2SO_4, filtered, and evaporated to afford compound 14b ((Z)-2-(3-(*tert*-butyldimethylsilyloxy)prop-1-enyl)phenyl propiontate) as a white powder.

YIELD:	443 mg (69.5 %)
HPLC:	R_t: 17.6 minutes (G1)
^{1}H NMR (300 MHz, CD_3OD):	δ 0.01 (s, 6H), 0.88 (s, 9H), 1.21 (t, 3H, J=7.5Hz), 2.58 (q, 2H, J=7.5Hz), 4.29 (dd, 2H, J=1.6Hz, J=6.3Hz), 5.87 (dt, 1H, J=6.3Hz, J=11.7Hz), 6.39 (dt, 1H, J=1.5Hz, J=11.6Hz), 7.06 (d, 1H, J=7.8Hz), 7.23-7.31 (m, 3H)
^{13}C NMR (75 MHz, CD_3OD):	δ -4.99, 9.47, 19.12, 26.42, 28.29, 61.36, 123.51, 125.33, 126.72, 129.70, 130.98, 131.36, 134.87, 147.87, 174.13

6.6.8 Synthesis of compound 15a,

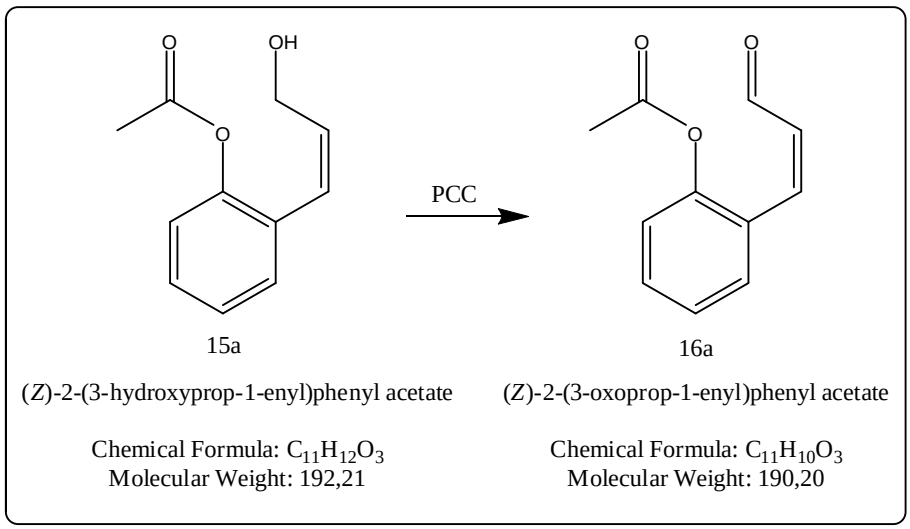
(Z)-2-(3-hydroxyprop-1-enyl)phenyl acetate [63]



To a solution of compound 14a (1.429 g, 4.7 mmol) in THF (13 ml) was added water (13 ml). This was followed by the drop wise addition of acetic acid (39 ml). The mixture was stirred at room temperature for 1 h and then evaporated to remove THF, water and acetic acid. Ethyl acetate (52.4 ml) was added to the residue, which was washed with 5 % $NaHCO_3$ (2 x 26 ml) and water (2 x 26 ml). The ethyl acetate solution was dried over Na_2SO_4, filtered, and evaporated. The residue was then re-dissolved in ACN and purified by preparative HPLC with ACN/HCOOH (aq) (20:80) to afford compound 15a ((Z)-2-(3-hydroxyprop-1-enyl)phenyl acetate) as a colorless oil.

YIELD:	48 mg (51.4 %)
HPLC:	R_t: 4.8 minutes (G1)
^{1}H NMR (300 MHz, CD_3OD):	δ 2.28 (s, 3H), 4.23 (dd, 2H, J=1.4Hz, J=6.7Hz), 5.96 (dt, 1H, J=6.7Hz, J=11.5Hz), 6.47 (d, 1H, J=11.5Hz), 7.05 (dd, 1H, J=1.0Hz, J=7.9Hz), 7.25 (m, 3H)
^{13}C NMR (75 MHz, CD_3OD):	δ 20.76, 59.85, 123.54, 125.32, 125.62, 126.81, 126.86, 128.10, 129.72, 149.92, 170.78

6.6.9 Synthesis of compound 16a, (Z)-2-(3-oxoprop-1-enyl)phenyl acetate [63]



15a

(*Z*)-2-(3-hydroxyprop-1-enyl)phenyl acetate

Chemical Formula: $C_{11}H_{12}O_3$
Molecular Weight: 192,21

16a

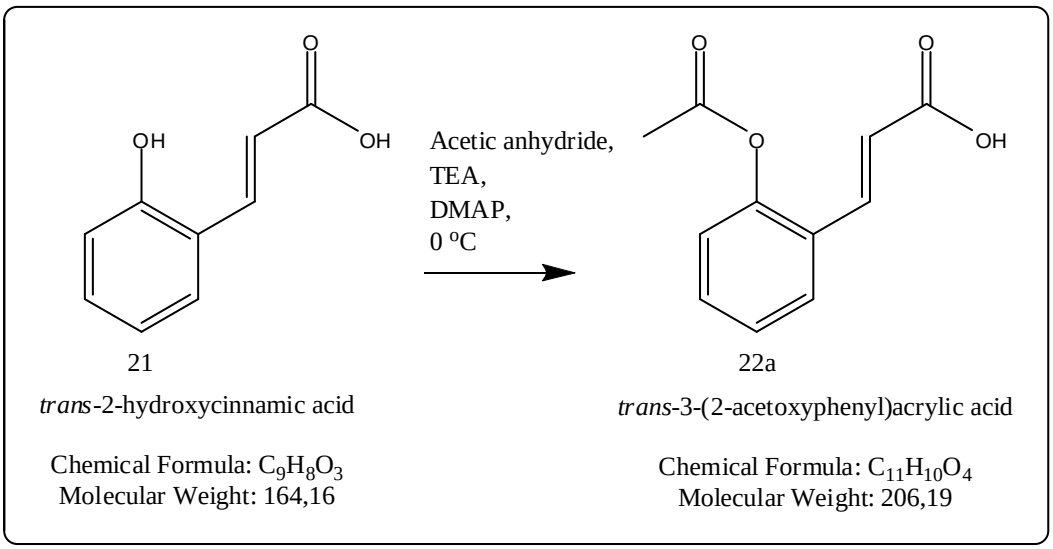
(*Z*)-2-(3-oxoprop-1-enyl)phenyl acetate

Chemical Formula: $C_{11}H_{10}O_3$
Molecular Weight: 190,20

To a solution of pyridinium chlorochromate (PCC) (3.9 g, 18.1 mmol) in dry CH_2Cl_2 (201 ml) was added drop wise a solution of compound 15a (1.74 g, 9.1 mmol) in dry CH_2Cl_2 (115 ml) during 20 min. After stirring at room temperature under Ar atmosphere for 30 min, the black solution was poured onto a short silica gel column and eluted with CH_2Cl_2 to remove the chromium salts. The black residue was washed several times with CH_2Cl_2 and the solution was run through the same column. The solution was then evaporated. The residue was then re-dissolved in ACN and purified by preparative HPLC with ACN/HCOOH (aq) (25:75) to afford compound 16a ((Z)-2-(3-oxoprop-1-enyl)phenyl acetate) as a green oil.

YIELD:	298 mg (17.3 %)
HPLC:	R_t: 7.3 minutes (G1)
^{1}H NMR (200 MHz, $CDCl_3$):	δ 2.29 (s, 3H), 6.21 (dd, 1H, J=8.1Hz, J=11.5Hz), 7.17 (dd, 1H, J=8.0Hz), 7.32-7.52 (m, 4H), 9.81 (d, 1H, J=8.1Hz)

6.6.10 Synthesis of compound 22a, *trans*-3-(2-acetoxyphenyl)acrylic acid [80]



21

trans-2-hydroxycinnamic acid

Chemical Formula: $C_9H_8O_3$
Molecular Weight: 164,16

22a

trans-3-(2-acetoxyphenyl)acrylic acid

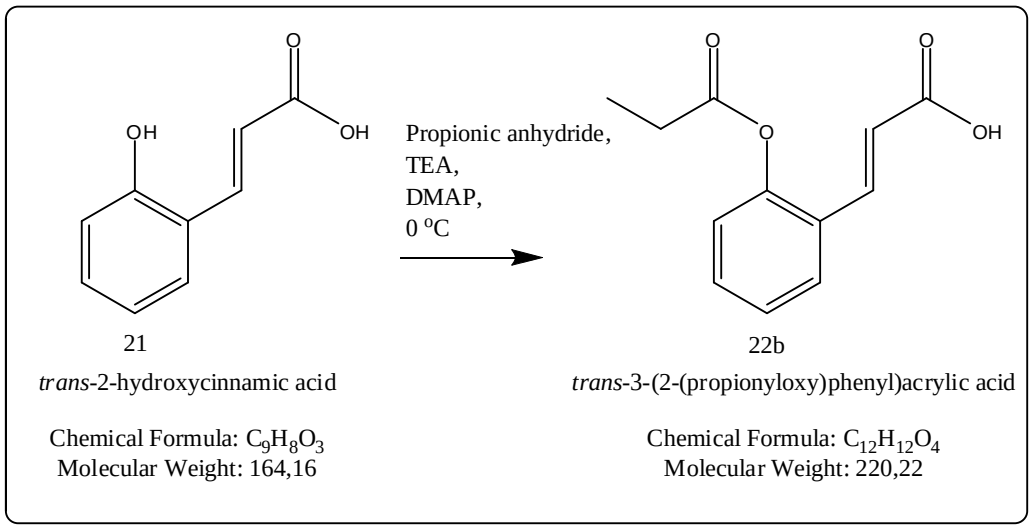
Chemical Formula: $C_{11}H_{10}O_4$
Molecular Weight: 206,19

Acetic anhydride (299 mg, 2.9 mmol) was added drop wise to a cooled (0 °C) solution of compound 21 (400 mg, 2.4 mmol) in anhydrous THF (20 ml) under Ar atmosphere with stirring. TEA (541 mg, 5.3 mmol) and DMAP (44 mg, 0.36 mmol) were added successively. The mixture was allowed to warm to room temperature and then stirred for another 2 h. After solvent evaporation, the residue was dissolved in ethyl acetate (53.5 ml) and washed with 1 M HCl (2 × 10 ml). The combined aqueous layer was then extracted with ethyl acetate. The organic layers were combined and washed with brine and then dried over $MgSO_4$. The solution was then filtered and evaporated to afford compound 22a (*trans*-3-(2-acetoxyphenyl)arylic acid) as a white solid.

YIELD:	450 mg (89.6 %)
HPLC:	R_t: 6.5 minutes (G1)
^{1}H NMR (300 MHz, DMSO):	δ 2.35 (s, 3H), 6.56 (d, 1H, J=16.1Hz), 7.21 (dd, 1H, J=0.8, J=8.1Hz), 7.32 (t, 1H, J=7.4Hz), 7.47 (m, 1H), 7.54 (d, 1H, J=16.1Hz), 7.87 (dd, 1H, J=1.1Hz, J=7.7Hz), 12.52 (s, 1H)
^{13}C NMR (75 MHz, DMSO):	δ 20.54, 121.29, 123.23, 126.35, 126.60, 127.68, 131.18, 136.53, 148.89, 167.13, 169.00

6.6.11 Synthesis of compound 22b,

trans-3-(2-(propionyloxy)phenyl)acrylic acid [80]



21

trans-2-hydroxycinnamic acid

Chemical Formula: $C_9H_8O_3$
Molecular Weight: 164,16

22b

trans-3-(2-(propionyloxy)phenyl)acrylic acid

Chemical Formula: $C_{12}H_{12}O_4$
Molecular Weight: 220,22

Propionic anhydride (285 mg, 2.2 mmol) was added drop wise to a cooled (0 °C) solution of compound 21 (300 mg, 1.8 mmol) in anhydrous THF (22.5 ml) under Ar atmosphere with stirring. TEA (407 mg, 4.0 mmol) and DMAP (36 mg, 0.29 mmol) were added successively. The mixture was allowed to warm to room temperature and then stirred for another 2 h. After solvent evaporation, the residue was dissolved in ethyl acetate (40 ml) and washed with 1 M HCl (2 × 10 ml). The combined aqueous layer was then extracted with ethyl acetate. The organic layers were combined and washed with brine and then dried over $MgSO_4$. The solution was then filtered and evaporated to afford compound 22b (*trans*-3-(2-(propionyloxy)phenyl)acrylic acid) as a white solid.

YIELD: 334 mg (83.0 %)

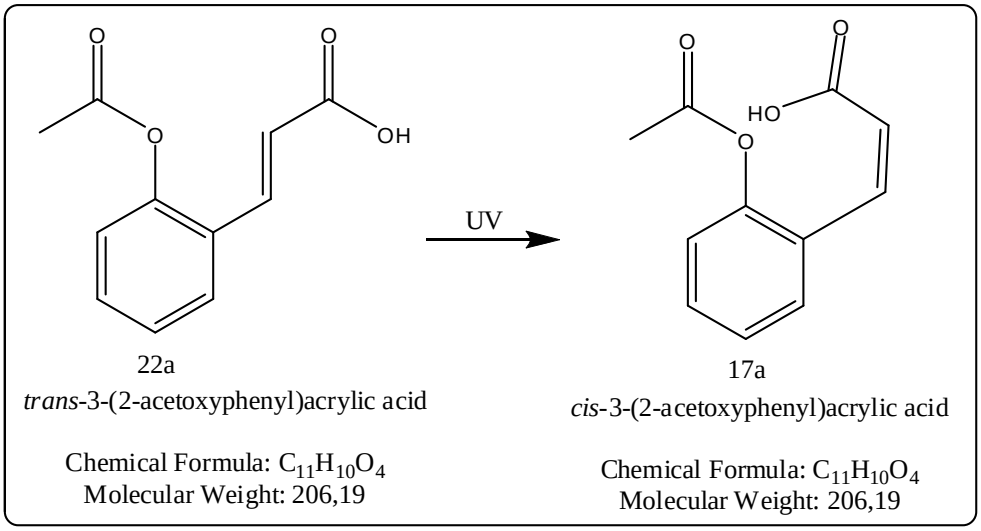
HPLC: R_t: 7.3 minutes (G1)

^{1}H NMR (300 MHz, DMSO): δ 1.18 (t, 3H, J=7.5Hz), 2.68 (q, 2H, J=7.5Hz), 6.55 (d, 1H, J=16.1Hz), 7.21 (dd, 1H, J=1.1Hz, J=8.1Hz), 7.32 (dt, 1H, J=0.7Hz, J=7.4Hz), 7.47 (m, 1H), 7.54 (d, 1H, J=16.1Hz), 7.87 (dd, 1H, J=1.5Hz, J=7.8Hz), 12.46 (s, 1H)

^{13}C NMR (75 MHz, DMSO): δ 8.89, 26.83, 121.25, 123.20, 126.32, 126.62, 127.68, 131.17, 136.53, 148.91, 167.12, 172.33

6.6.12 Synthesis of compound 17a,

cis-3-(2-acetoxyphenyl)arylic acid [80]



22a
trans-3-(2-acetoxyphenyl)acrylic acid

Chemical Formula: $C_{11}H_{10}O_4$
Molecular Weight: 206,19

17a
cis-3-(2-acetoxyphenyl)acrylic acid

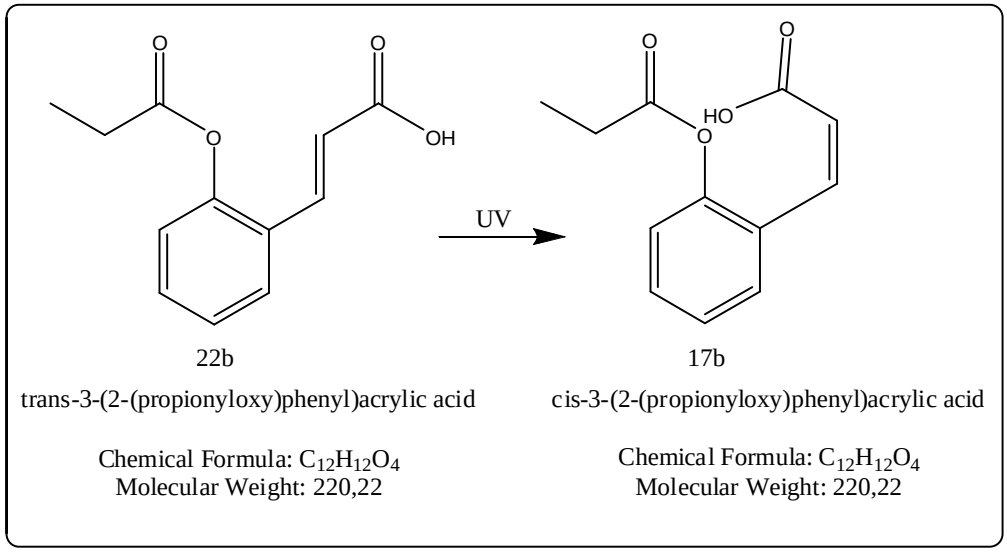
Chemical Formula: $C_{11}H_{10}O_4$
Molecular Weight: 206,19

Compound 22a (314 mg, 2.2 mmol) was weight out in a 100 ml round bottomed flask and dissolved in methanol (50 ml). The solution was placed under an UV-lamp for irradiation at 365 nm for 3 days. The solution was then evaporated and the residue was re-dissolved in ACN and purified by preparative HPLC with ACN/HCOOH (aq) (25:75) to afford compound 17a (*cis*-3-(2-acetoxyphenyl)arylic acid) as a white solid.

YIELD:	40 mg (12.7 %)
HPLC:	R_t: 6.0 minutes (G1)
^{1}H NMR (300 MHz, CD_3OD):	δ 2.26 (s, 3H), 6.06 (d, 1H, J=12.4Hz), 6.88 (d, 1H, J=12.4Hz), 7.08-7.53 (m, 4H)
^{13}C NMR (75 MHz, CD_3OD):	δ 20.81, 123.29, 124.42, 126.55, 130.14, 130.55, 131.25, 136.98, 149.74, 169.85, 170.76
MS (ES^+; TOF):	m/z 229.1 ($[M+Na]^+$ = 100 %), 251.1 (23 %)

6.6.13 Synthesis of compound 17b,

cis-3-(2-(propionyloxy)phenyl)acrylic acid [80]



Compound 22b (334mg, 1.52 mmol) was weight out in a 100 ml round bottomed flask and dissolved in methanol (50 ml). The solution was placed under an UV-lamp for irradiation at 365 nm for 5 days. The solution was then evaporated and the residue was re-dissolved in ACN and purified by preparative HPLC with ACN/HCOOH (aq) (30:70) to afford compound 17b (*cis*-3-(2-propionyloxy)phenyl)arylic acid) as a white solid.

YIELD: 69 mg (20.7 %)

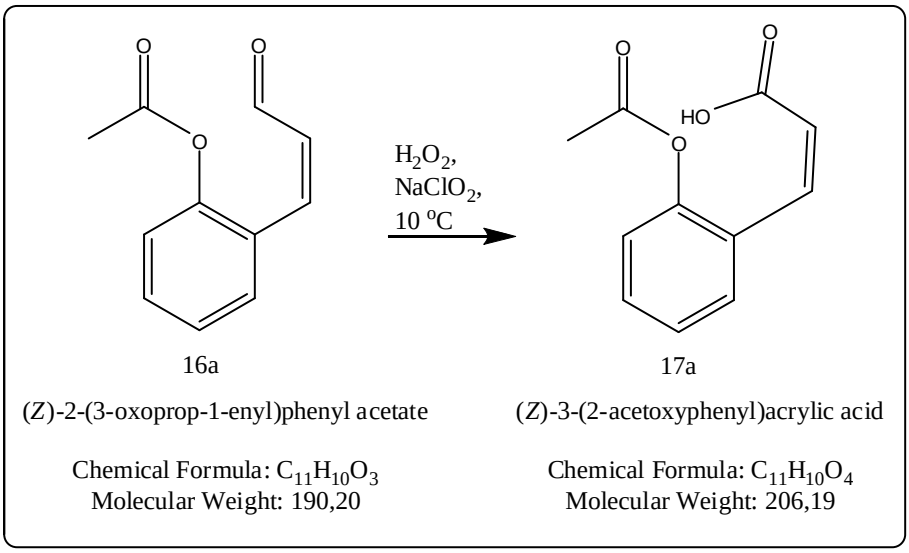
HPLC: R_t: 7.1 minutes (G1)

^{1}H NMR (300 MHz, DMSO): δ 1.13 (t, 3H, J=7.5Hz), 2.58 (q, 2H, J=7.5Hz), 6.03 (d, 1H, J=12.4Hz), 6.84 (d, 1H, J=12.4Hz), 7.13 (dd, 1H, J=1.1Hz, J=8.1Hz), 7.23 (dt, 1H, J=1.0Hz, J=7.5Hz), 7.37 (dt, 1H, J=1.6Hz, J=7.7Hz), 7.53 (dd, 1H, J=1.4Hz, J=7.7Hz), 12.43 (s, 1H)

^{13}C NMR (75 MHz, DMSO): δ 8.74, 26.69, 122.19, 123.38, 125.19, 128.25, 129.44, 129.87, 134.97, 147.96, 166.77, 171.99

6.7 ATTEMPTED SYNTHESIS

6.7.1 Attempted synthesis of compound 17a, (Z)-3-(2-acetoxyphenyl)arylic acid [63]



16a

(*Z*)-2-(3-oxoprop-1-enyl)phenyl acetate

Chemical Formula: $C_{11}H_{10}O_3$
Molecular Weight: 190,20

17a

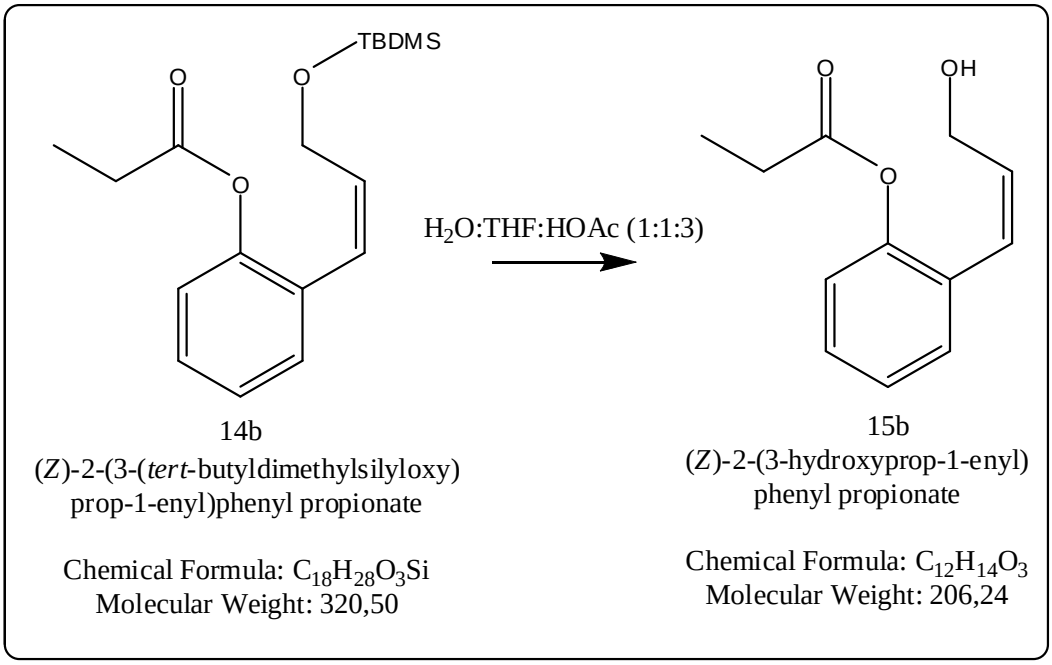
(*Z*)-3-(2-acetoxyphenyl)acrylic acid

Chemical Formula: $C_{11}H_{10}O_4$
Molecular Weight: 206,19

A solution of 80% sodium chlorite (379 mg, 4.2 mmol) in water (3.35 ml) was added drop wise to a stirred mixture of (Z)-2-(3-oxoprop-1-enyl)phenyl acetate (454 mg, 2.4 mmol) in ACN (2.39 ml), sodium phosphate (77 mg, 0.5 mmol) in water (0.96 ml), and 30 % hydrogen peroxide (0.28 ml). During the addition, the reaction temperature was kept at 10 °C with an ice-water bath. Oxygen evolution from the solution was observed visually until the end of the reaction. A small amount of sodium sulfite was added to destroy the unreacted HOCl and H_2O_2. The solution was acidified with 1 M HCl to pH 1- 2. The mixture was then extracted with ethyl acetate (47 ml). The ethyl acetate layer was washed with saturated sodium chloride solution (2 x 18 ml) and dried over Na_2SO_4 and filtered. The filtrate was evaporated and the residue re-dissolved in ACN. The solution was then purified by preparative HPLC with ACN/HCOOH (aq) (25:75) to afford a white solid (20 mg).

After analyzing NMR-spectra no reaction product could be detected.

6.7.2 Attempted synthesis of compound 15b, (Z)-2-(3-hydroxyprop-1-enyl)phenyl propionate [63]



14b
(*Z*)-2-(3-(*tert*-butyldimethylsilyloxy) prop-1-enyl)phenyl propionate

Chemical Formula: $C_{18}H_{28}O_3Si$
Molecular Weight: 320,50

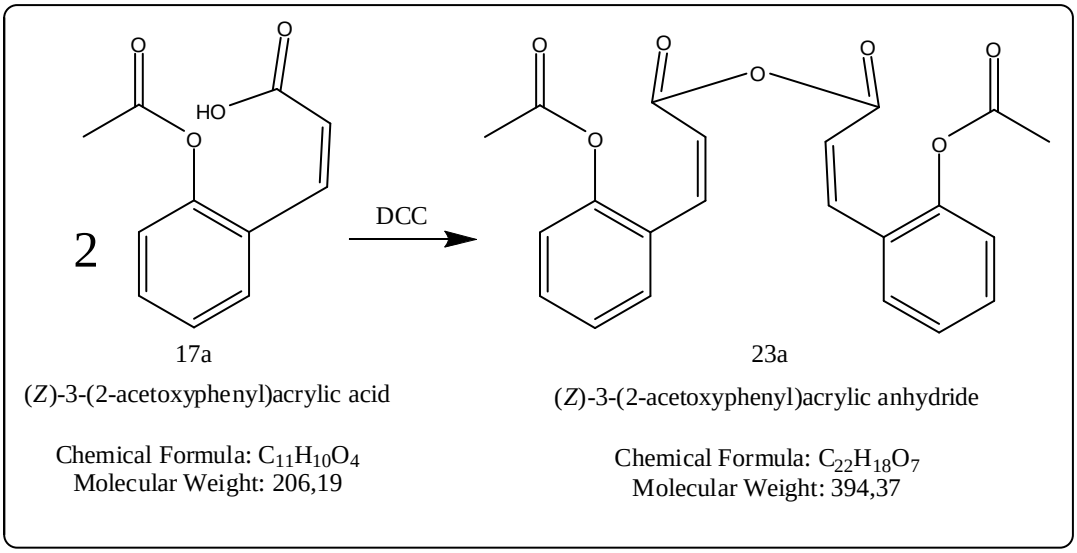
15b
(*Z*)-2-(3-hydroxyprop-1-enyl) phenyl propionate

Chemical Formula: $C_{12}H_{14}O_3$
Molecular Weight: 206,24

To a solution of compound 14b (443 mg, 1.38 mmol) in THF (8 ml) was added water (8 ml). This was followed by the drop wise addition of acetic acid (24 ml). The mixture was stirred at room temperature for 3 h and then evaporated to remove THF, water and acetic acid. Ethyl acetate (15 ml) was added to the residue, which was washed with 5 % $NaHCO_3$ (2 x 8 ml) and water (2 x 8 ml). The ethyl acetate solution was dried over Na_2SO_4, filtered, and evaporated.

The peak for compound 14b in the HPLC chromatogram was gone and analysis of NMR spectra could not detect any product.

6.7.3 Attempted synthesis of compound 23a, (Z)-3-(2-acetoxyphenyl)arylic anhydride



17a
(*Z*)-3-(2-acetoxyphenyl)acrylic acid

Chemical Formula: $C_{11}H_{10}O_4$
Molecular Weight: 206,19

23a
(*Z*)-3-(2-acetoxyphenyl)acrylic anhydride

Chemical Formula: $C_{22}H_{18}O_7$
Molecular Weight: 394,37

A solution of compound 17a ((Z)-3-(2-acetoxyphenyl)arylic acid) (156 mg, 0.8 mmol) in DCM (10 ml) was placed on an ice bath. The cold solution was added dicyclohexylcarbodiimide (DCC) (78 mg, 0.4 mmol) and stirred for 30 minutes. Then the mixture was allowed to warm to room temperature and then stirred for another 24 h.

Method 1:

The solution was then filtered and carefully evaporated to give a colorless oil.

^{1}H and ^{13}C NMR: no signal from the product shown

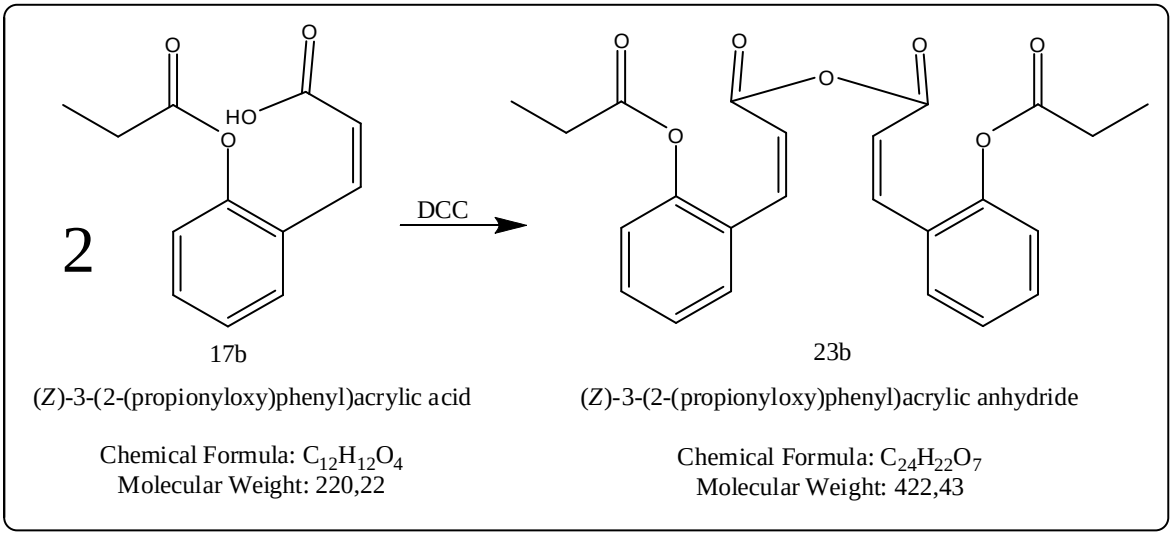
Method 2:

The solution was then cold down to 0°C and allowed to stir for 30 minutes. The cold solution was filtered and carefully evaporated to give a colorless oil.

^{1}H and ^{13}C NMR: no signal from the product shown

6.7.4 Attempted synthesis of compound 23b,

(Z)-3-(2-(propionyloxy)phenyl)acrylic anhydride



A solution of compound 17b ((Z)-3-(2-(propionyloxy)phenyl)arylic acid) (126 mg, 0.572 mmol) in DCM (10 ml) was placed on an ice bath. The cold solution was added dicyclohexylcarbodiimide (DCC) (59 mg, 0.29 mmol) and stirred for 30 minutes. Then the mixture was allowed to warm to room temperature and then stirred for another 24 h.

Method 1:

The solution was then filtered and carefully evaporated to give a colorless oil.

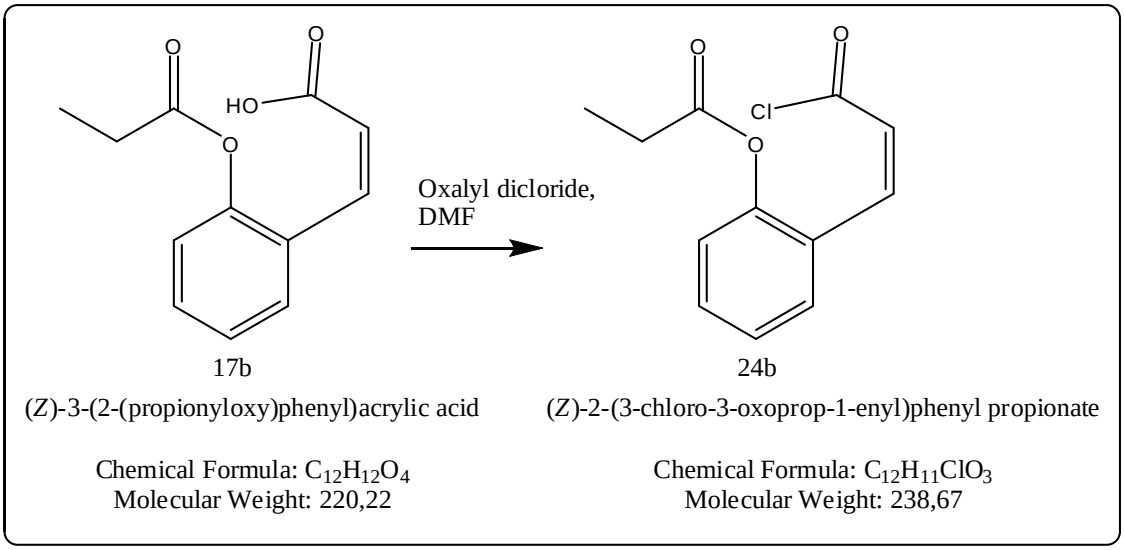
^{1}H and ^{13}C NMR: no signal from the product shown

Method 2:

The solution was then cold down to 0°C and allowed to stir for 30 minutes. The cold solution was filtered and carefully evaporated to give a colorless oil.

^{1}H and ^{13}C NMR: no signal from the product shown

6.7.5 Attempted synthesis of compound 24b, (Z)-2-(3-chloro-3-oxoprop-1-enyl)phenyl propionate



17b
(*Z*)-3-(2-(propionyloxy)phenyl)acrylic acid

Chemical Formula: $C_{12}H_{12}O_4$
Molecular Weight: 220,22

24b
(*Z*)-2-(3-chloro-3-oxoprop-1-enyl)phenyl propionate

Chemical Formula: $C_{12}H_{11}ClO_3$
Molecular Weight: 238,67

To a solution of compound 17b ((Z)-3-(2-(propionyloxy)phenyl)acrylic acid) (51 mg, 0.23 mmol) in dichloromethane (5 ml) was added 1 droplet of N,N-dimethylformamide (DMF). The solution was placed on a magnetic stirrer with a condenser. A surplus of oxalyl chloride was drop wise added, approximately 0.5 – 1 ml. Gas evolution of the solution was visually observed. The solution was carefully evaporated and analyzed by HPLC.

No peak from the methyl ester of compound 24b observed in the HPLC chromatogram.

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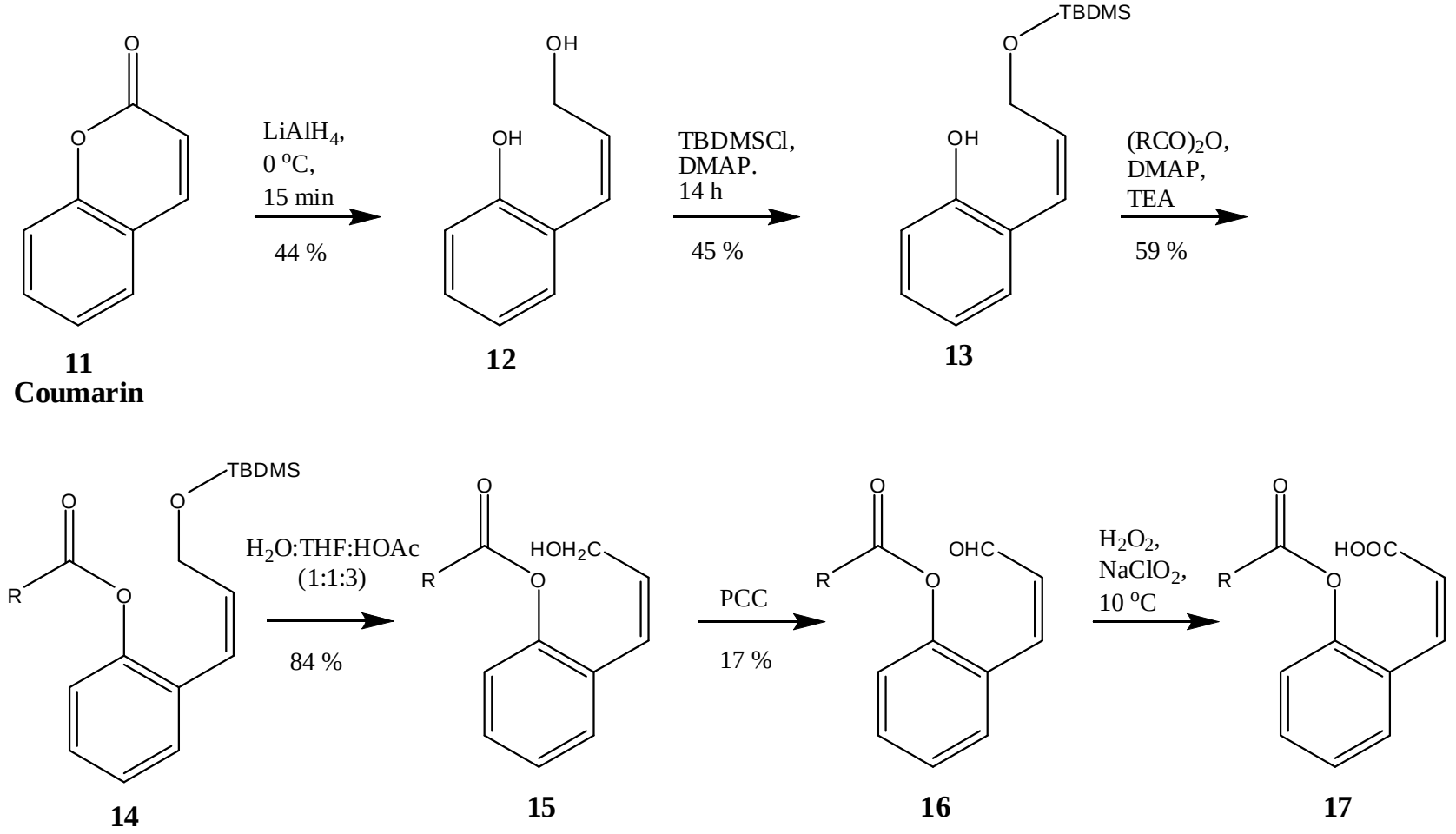
APPENDIX A Synthesis from coumarin



11
Coumarin

12

13

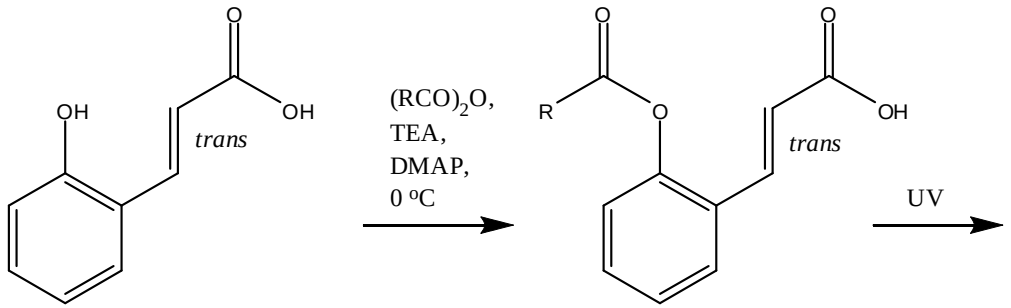
14

15

16

17

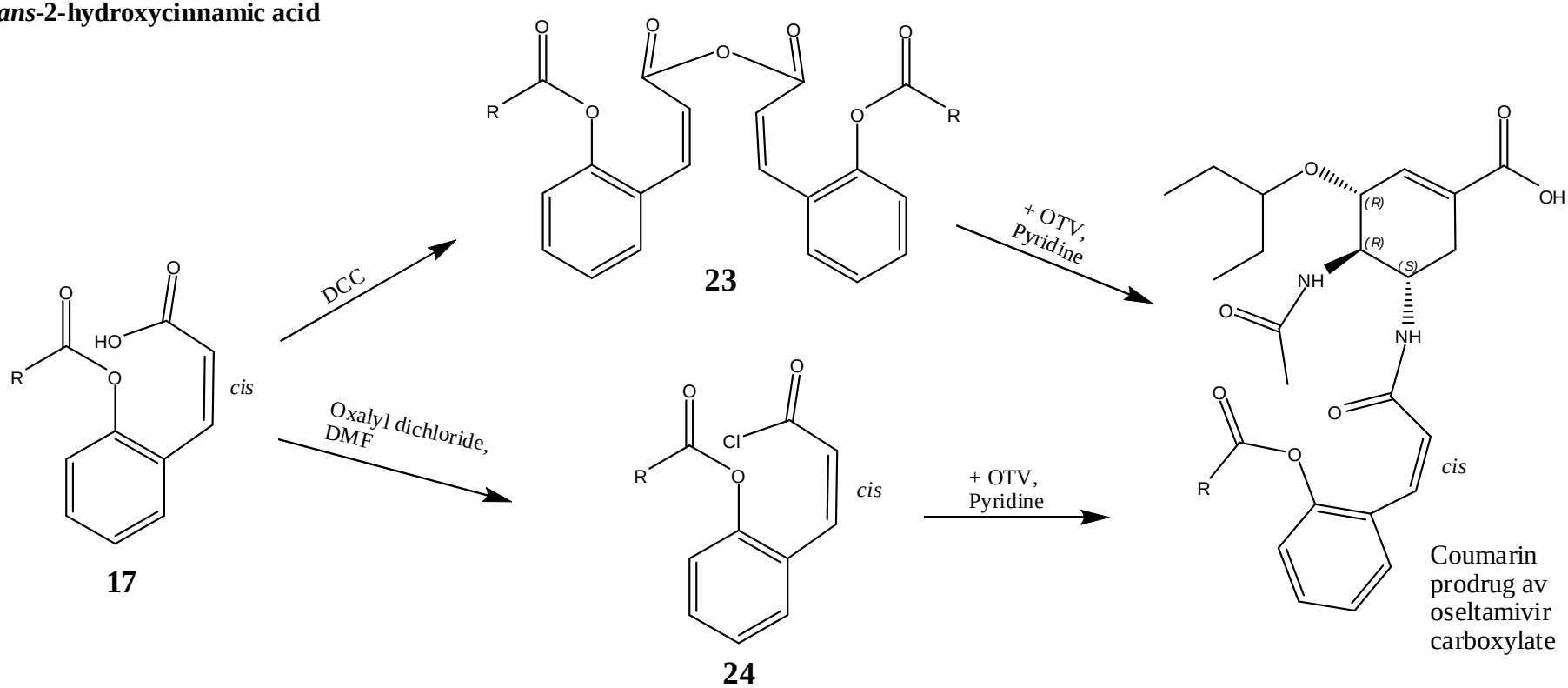
APPENDIX B Synthesis from *trans*-2-hydroxycinnamic acid



21

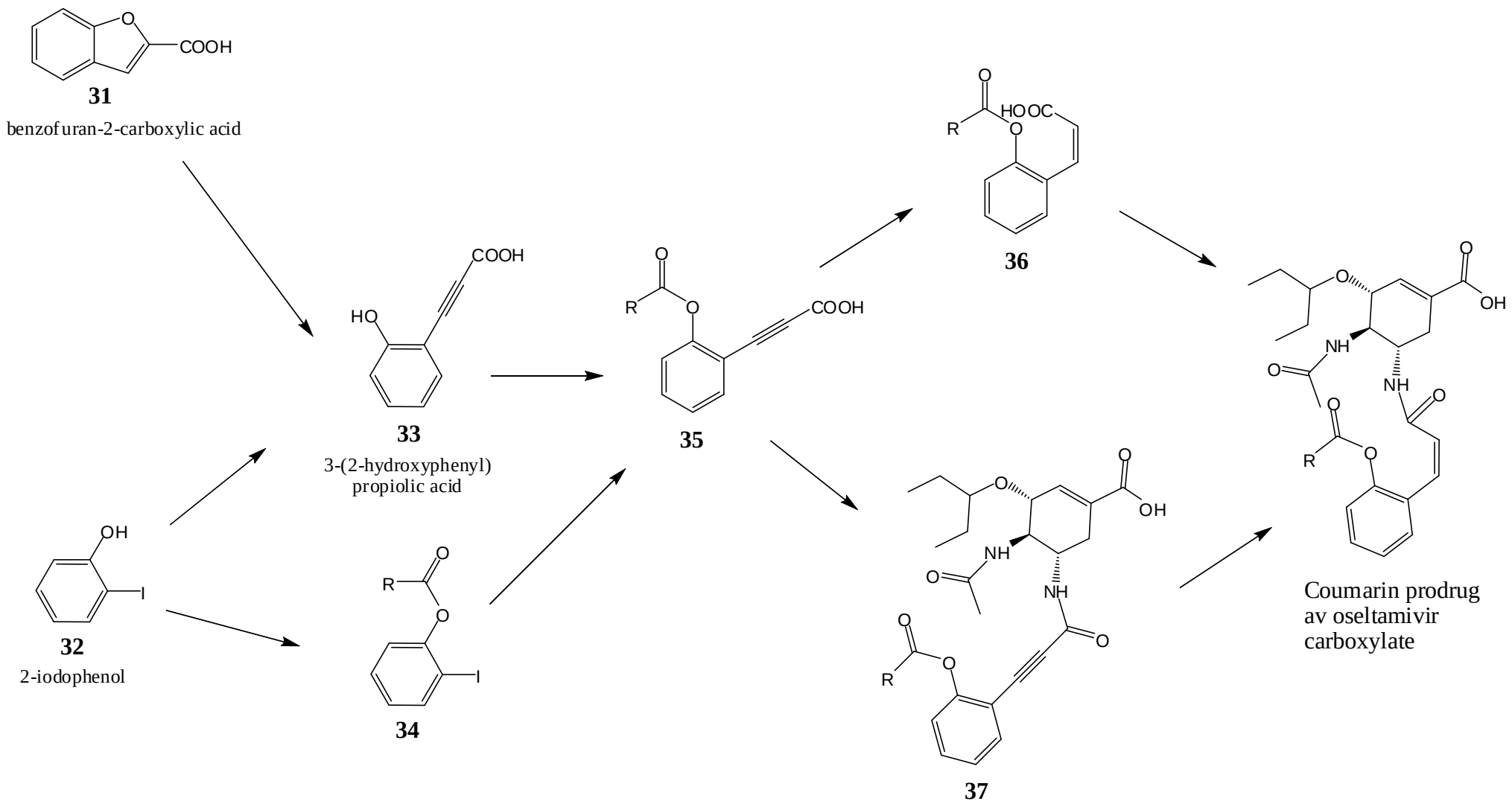
***trans*-2-hydroxycinnamic acid**

22



Coumarin prodrug av oseltamivir carboxylate

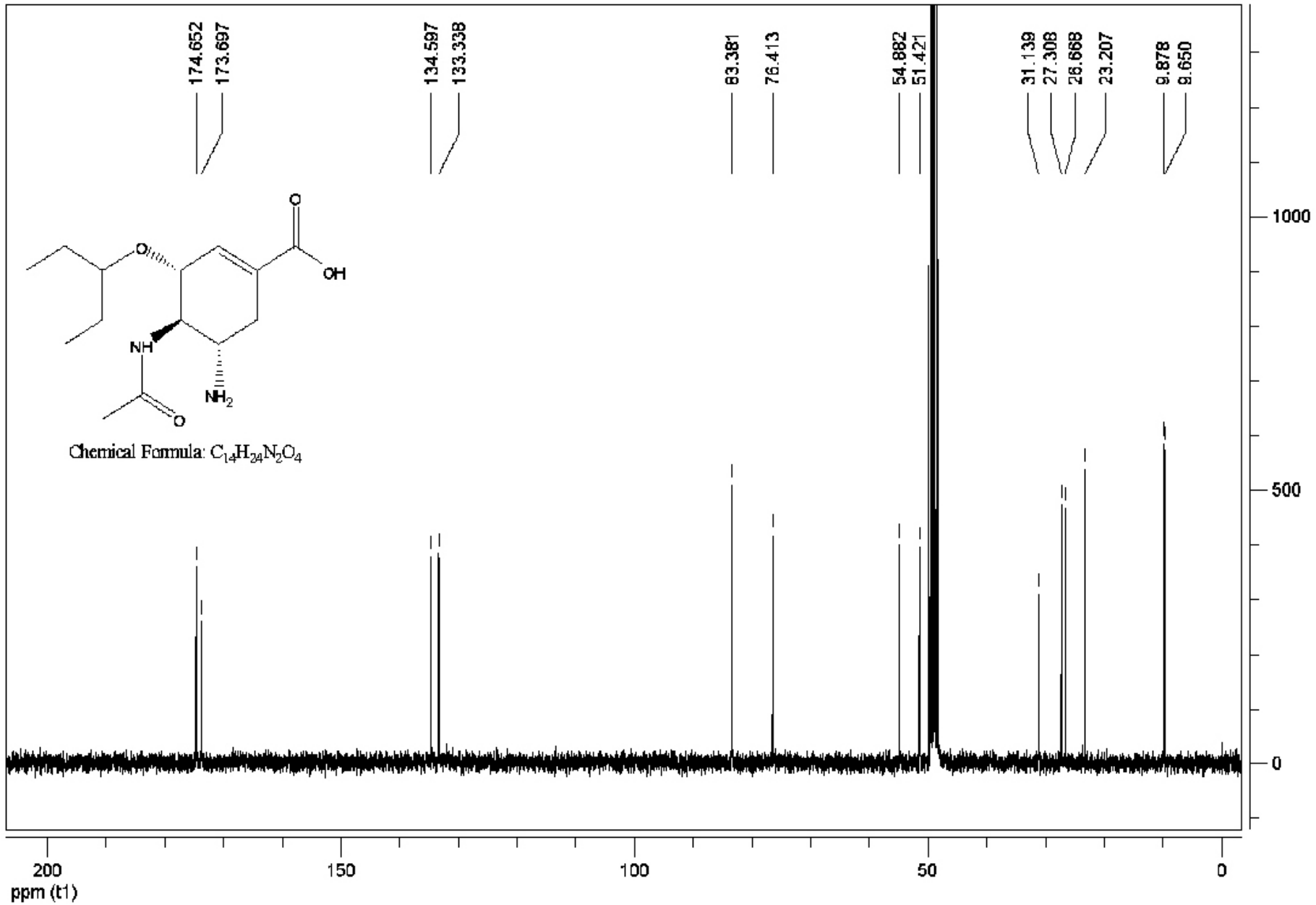
APPENDIX C Catalytical hydrogenation of an alkyne

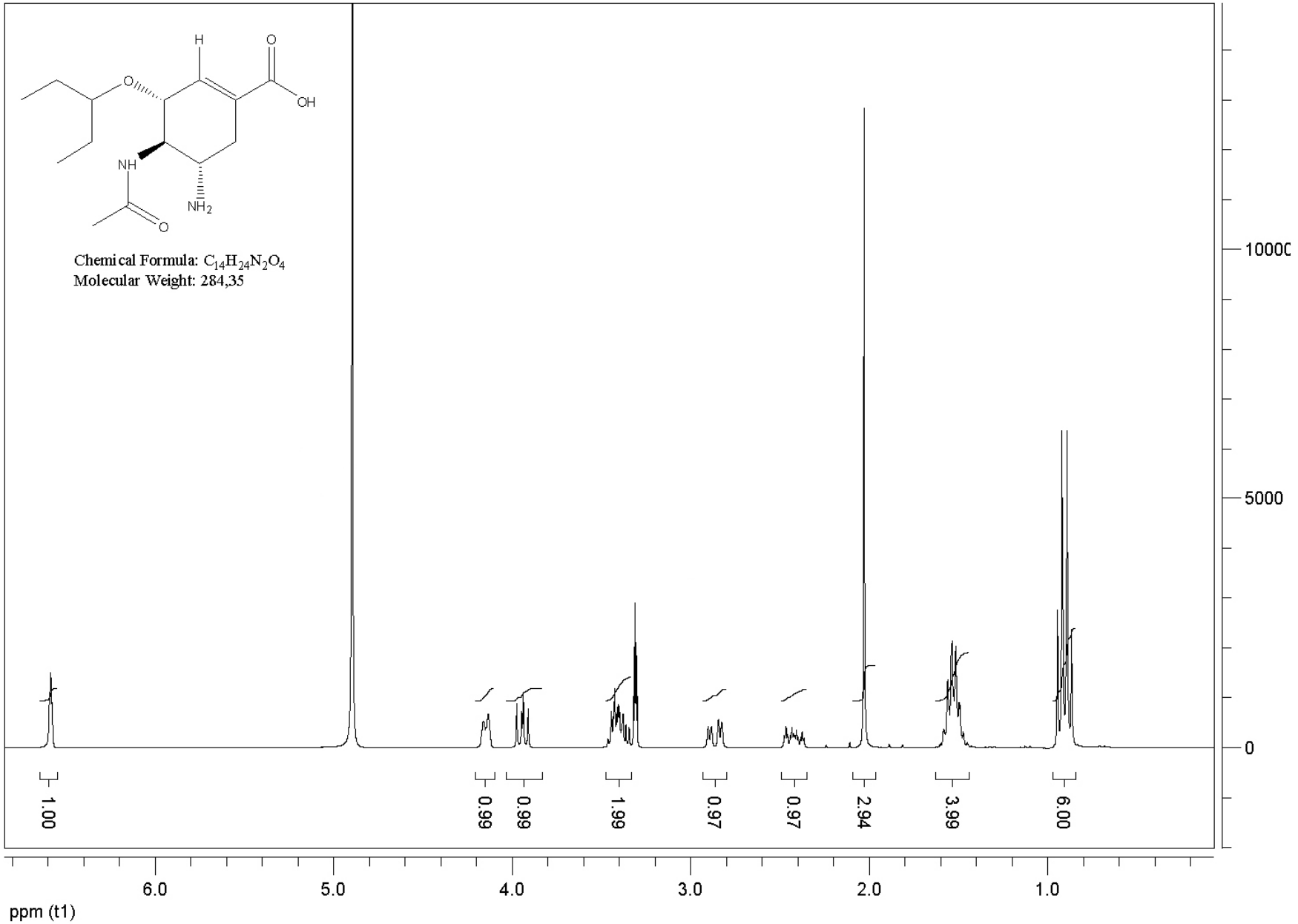


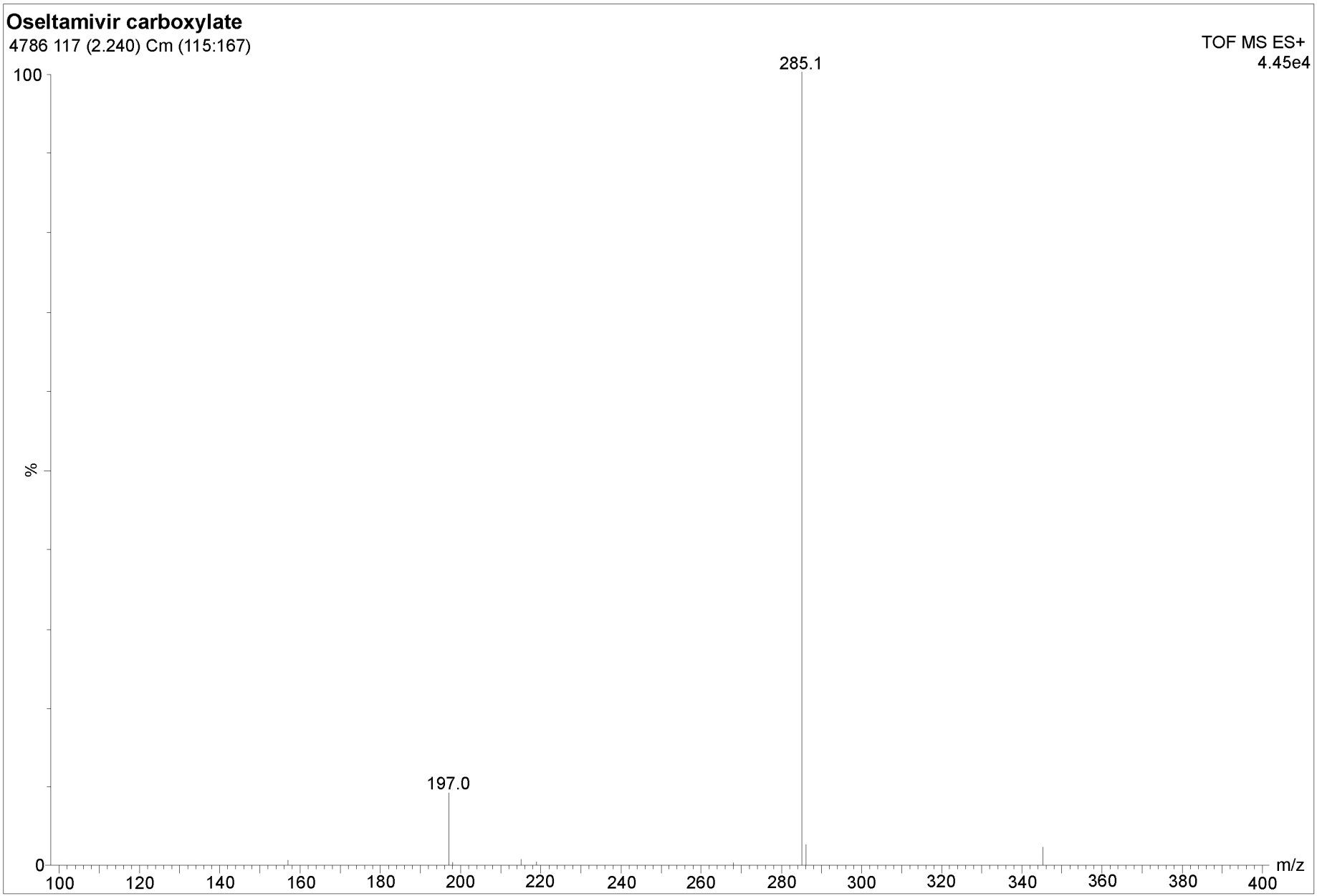
APPENDIX D

NMR and MS spectra

for oseltamivir carboxylate


174.652
173.697
134.597
133.338
83.381
76.413
54.882
51.421
31.139
27.308
26.668
23.207
9.878
9.650
O
OH
O
NH
NH2
O
Chemical Formula: C14H24N2O4
1000
500
0
200
150
100
50
0
ppm (t1)


Chemical Formula: $C_{14}H_{24}N_2O_4$
Molecular Weight: 284,35
10000
5000
0
1.00
0.99
0.99
1.99
0.97
0.97
2.94
3.99
6.00
6.0
5.0
4.0
3.0
2.0
1.0
ppm (t1)


Oseltamivir carboxylate
4786 117 (2.240) Cm (115:167)
TOF MS ES+
4.45e4
285.1
197.0
100
%
0
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
m/z

APPENDIX E Chemicals and solvents for synthesis

	Quality	Supplier
Acetonitrile	≥ 99.9 %	Merck
Absolutt alkohol prima	-	Arcus
Acetic acid	> 90 %	Merck
Acetic acid anhydride	> 98 %	KEBO Lab
Acetone	> 99.5 %	Merck
Ammonium acetate	≥ 96 %	BDH Laboratory chemicals
Coumarin	-	Sigma
Dichloromethane	> 99.9 %	Fluka, Riedel-de Häen
N,N-dicyclohexylcarbodiimide	~ 99 %	Fluka
Diethyl ether	min 99.5 %	Riedel-de Häen
N,N-Dimethylformamide	≥ 99.8 %	Fluka, Riedel-de Häen
N,N-Dimethylpyridin-4-amine	≥ 98 %	Fluka
Ethyl acetate	> 99.5 %	Fluka
Formic acid	98-100 %	Merck
Hydrochloric acid 32 %	min 32 %	Riedel-de Häen
Hydrogen peroxide 30%	≥ 30 %	Fluka
Hydroxylamine Solution	~ 50 %	Fluka
Isopropanol	-	A/S Vinmonopolet
Lithium aluminum hydride solution	-	Aldrich
Magnesium sulfate anhydrous	≥ 98 %	Fluka
Methanol	min 99.9 %	Sigma-Aldrich
Oxalyl chloride	~ 98 %	Fluka
Propionic anhydride	~ 98 %	Fluka
Pyridine	≥ 99.8 %	Fluka
Pyridinium chlorochromate	≥ 98 %	Fluka
Sodium bicarbonate	≥ 99.5 %	Aldrich
Sodium carbonate	-	KEBO Lab
Sodium chlorite	80 %	Fluka
Sodium phosphate	96 %	Aldrich
Sodium sulfite	-	KEBO Lab
Sodium sulphate	≥ 99.0 %	Fluka
tert-Butyl-dimethylsilylchlorid	97 %	Aldrich
Tetrahydrofuran	min 99.9 %	Sigma-Aldrich
Tetrahydrofuran, anhydrous	≥ 99.9 %	Fluka
trans-O-coumaric acid	>98 %	TCI Europe
Triethylamine	98 %	Fluka